Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 75 DECEMBER, 1950 No. 3

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Effects of Amphenone "B"* on Thyroid, Adrenals, and Genital Tract of the Female Rat. (18286)

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We have previously described the progesterone-like effect of Amphenone "B"* on the rabbit(1). The present report will describe the marked adrenal and thyroid hypertrophy as well as the trophic effects on the genital tract exerted by this compound in the female rat (Fig. 1).

Materials and methods. Amphenone "B" was synthesized by the method of Allen and Corwin(2). The compound was stored in a vacuum desiccator and for administration dissolved in triple-distilled water not more than 3 days before use. Solutions were stored in the refrigerator when not in use. The

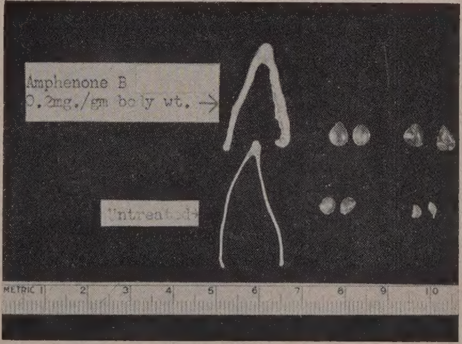

FIG. 1.
Uterus, adrenals, and thyroid of treated and untreated ovariectomized rats.

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* 1,2-bis-(p-Aminophenyl)-2-Methylpropanone-1 Dihydrochloride.

1. Allen, Hertz, and Tullner, PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 632.

2. Allen and Corwin, *J.A.C.S.*, 1950, v72, 117.

TABLE I. Effect of Amphenone ''B'' on Adrenal, Uterine, and Thymus Weight.

Series	Age at ovariectomy (days)	Age at autopsy (days)	No. of rats	No. of rats surviving	Dose* in mg	Body wt at autopsy (g)	Adrenal wt (mg)	Uterine wt (mg)	Thymus wt (mg)
A	49	112	10	10	50	208 ± 17	164 ± 42	101 ± 27	226 ± 49
”	”	”	”	9	45	226 ± 14	151 ± 18	102 ± 14	222 ± 21
”	”	”	”	10	40	231 ± 9	139 ± 23	92 ± 23	249 ± 29
”	”	”	”	9	30	244 ± 14	116 ± 17	78 ± 20	373 ± 157
”	”	”	”	10	25	252 ± 7	103 ± 10	75 ± 15	373 ± 62
”	”	”	”	10	0	260 ± 31	59 ± 3	55 ± 7	539 ± 96
B	28	105	10	3	75	184 ± 1	175 ± 10	84 ± 9	172 ± 36
”	”	”	”	10	50	211 ± 15	188 ± 27	80 ± 12	245 ± 53
”	”	”	”	10	25	230 ± 10	98 ± 8	63 ± 8	359 ± 101
”	”	”	”	10	12.5	263 ± 8	76 ± 10	45 ± 6	565 ± 73
”	”	”	”	10	0	268 ± 15	67 ± 7	34 ± 8	648 ± 50
C	28	84	5	5	50	225 ± 12	205 ± 16	66 ± 6	236 ± 40
”	”	”	”	5	25	231 ± 8	108 ± 14	43 ± 7	391 ± 29
”	”	”	”	5	12.5	239 ± 6	86 ± 5	35 ± 6	499 ± 115
”	”	”	”	5	0	279 ± 19	70 ± 5	27 ± 7	818 ± 24

* Daily oral dose given in from 0.5 to 1.0 cc aqueous sol. for 5 consecutive days; autopsy 24 hr after last dose.

TABLE II. Effect of Amphenone ''B'' on Thyroid, Adrenal, and Uterine Weight.

Group	Treatment in addition to Amphenone ''B''	Days treated with Amphenone ''B''	Daily dose,§ mg	No. of animals	Age at autopsy (days)	Body wt at autopsy, g	Adrenal wt, mg	Uterine wt, mg	Thyroid wt, mg	Thymus wt, mg
I	Adrenalectomy† and ovariectomy*	3	40	10	93	234 ± 19		73 ± 13		749 ± 94
		0	0	3		217 ± 9		29 ± 4		972 ± 90
II	Cortisone‡ and ovariectomy*	4	40	6	91	185 ± 7	61 ± 5	70 ± 12	12 ± 3	91 ± 12
II(a)	Ovariectomy*	4	40	7		226 ± 9	129 ± 11	59 ± 5	15 ± 4	388 ± 96
III	Ovariectomy*	22	12	5	50	111 ± 5	75 ± 7	69 ± 17	30 ± 9	232 ± 23
		0	0	5		125 ± 10	40 ± 2	22 ± 3	9 ± 1	522 ± 94
IV	Ovariectomy*	15	38	4	87	182 ± 9	103 ± 10	145 ± 10	42 ± 11	288 ± 71
		0	0	4		238 ± 12	66 ± 6	27 ± 3	14 ± 2	748 ± 47
V	Ovariectomy*	16	18	5	79	175 ± 13	141 ± 20	58 ± 7	40 ± 4	284 ± 51
		0	0	5		219 ± 12	66 ± 13	30 ± 3	16 ± 3	686 ± 61

* Ovariectomized at 24 days of age.

† Adrenalectomized on day before starting Amphenone ''B''; maintained on saline for drinking water.

‡ 5 mg cortisone acetate given daily subcutaneously in 0.2 cc aqueous suspension beginning 2 days before Amphenone ''B''.

§ Amphenone ''B'' given daily in 0.5 to 1 cc aqueous solution by stomach tube.

daily dose was administered by stomach tube in from 0.5 to 1.0 cc of water. Female rats of the Holtzman strain were employed. Ovariectomy was done when the animals were 3 to 7 weeks of age. The animals were sacrificed by exposure to ether or chloroform and their organs were weighed to the nearest milligram on a Roller-Smith torsion balance. Tissues were fixed in 10% formalin and stained by routine hematoxylin and eosin.

Results and discussion. The genital tract of previously ovariectomized adult and juvenile rats exhibited an atypical trophic response to Amphenone "B" (Tables I, II; Fig. 1). The uteri were grossly enlarged and were distended with a mucoid secretion somewhat more viscous than that usually seen after estrogen administration. The vaginal membrane was broken down in most cases and a mucoid fluid could be readily expressed from the vagina. Microscopic examination of vaginal smears revealed a mixed smear consisting of nucleated and cornified epithelial cells in about equal numbers, leucocytes being only rarely seen. The uterine weights following any given dosage were quite variable and reached a maximum at about a 3- to 4-fold increment above the controls.

The adrenals of adult animals treated for 3 to 5 days showed a remarkable degree of hypertrophy (Tables I, II; Fig. 1). These enlarged adrenals have a normal yellow color and are readily differentiated from the red or brown adrenal seen in non-specific intoxications. Table I shows the quantitative relationship between the dose of drug and the degree of adrenal stimulation. This indicates that the effect is not a "trigger-response," but is quantitatively related to the amount of drug administered.

The daily ingestion of Amphenone "B" over a period of 15 to 22 days in the growing rat leads to a striking hypertrophy of the thyroid (Table II; Fig. 1). In some cases this represents a four-fold increment in thyroid weight. These enlarged glands are grossly hyperaemic and very friable.

The adrenal enlargement was absent in 7 hypophysectomized animals treated for 4 to 7 days with daily doses of 0.1 mg to 0.2 mg per gram of body weight of Amphenone "B". This indicates that the adrenotrophic effects are mediated through the pituitary gland. Moreover, the adrenal stimulation can be prevented by the simultaneous administration of cortisone which is known to suppress endogenous adrenotrophin formation(3),(Table II).

It is apparent that the hypertrophied adrenals are producing corticoids because the thymus involutes substantially in all animals showing adrenal stimulation following Amphenone "B" and such thymic involution is lacking in previously adrenalectomized animals treated with the Compound (Tables I, II). That the uterine hypertrophy seen in ovariectomized Amphenone "B"-treated animals is a direct effect on the genital tract is shown by the persistence of this response after either hypophysectomy or adrenalectomy (Table II).

Preliminary histological study of the grossly affected organs indicates that the adrenals retain essentially normal zonal relationships except for the compression of the medulla by the enlarged cortex. The cortical cells are laden with lipid and the individual cells are substantially enlarged.

The hyperplastic thyroids are almost colloid-free and the secretory epithelium is of a tall, columnar type. The vascular and lymph channels are widely dilated.

The acute toxicity of Amphenone "B" has been found to be quite high. Thus a dose of .4 mg/g body weight given orally in 0.5 cc H_2O will regularly kill adult female rats. Since its maximum effective dose in terms of uterine, thyroid, and adrenal stimulation is about 0.25 mg/g, the margin of safety of this compound must be considered fairly small. Moreover, the nature of the acute toxic effects is of some interest, since animals exposed to a sublethal dose show a profound degree of narcosis lasting for from 1 to 6 hours, after which the animal resumes normal behavior. At lethal doses this narcosis will persist for 10 to 12 hours preceding the animal's death.

It is apparent that Amphenone "B" not only exerts a marked trophic action on the genital tract of the ovariectomized rat, but that it also stimulates increased thyrotropic and adrenotropic activity in the anterior pituitary. These endocrine effects coupled with its depressant action on the central nervous system and its acute toxicity render Amphenone "B" and its analogues worthy of further biological and pharmacological investigation.

Summary. Amphenone "B" administered either orally or parenterally, exerts an atypical trophic action on the genital tract of the

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3. Sayers, G., *Phys. Rev.*, 1950, v30, 241.

ovariectomized female rat. It also effects a rapid hypertrophy of the adrenals and thyroid. These adrenal effects are lacking in the hypophysectomized rat but the uterine stimulation is unaffected either by hypophysectomy or adrenalectomy. The compound also possesses an acute toxicity which is characterized by marked narcosis leading to death. The maximum effective dose is approximately two-thirds of the lethal dose.

Received October 27, 1950. P.S.E.B.M., 1950, v75.

Failure of Cockerel Comb and Testis Development on Sesame Meal and its Prevention by Vitamin B_{12}.*† (18287)

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During the course of a nutrition experiment designed by one of us (A.R.P.), it was observed that when the ration contained 70% sesame meal there was marked failure of development of combs and testes. This condition was prevented by adding Vit. B_{12} to the diet. The source of the Vit. B_{12} was Merck APF Supplement No. 3.

Methods and materials. Single comb White Leghorn cockerels were fed a Vit. B_{12} depletion diet based on soy bean (sodium proteinate) which contained 0.05% iodinated casein (Protamone). These birds were distributed evenly by weight into groups of 15 each, eliminating extremes in weight. The experimental rations consisting of sesame meal 70%, yellow corn meal 26%, dicalcium phosphate 2%, pulverized limestone 1%, salt mixture 0.5%, choline chloride 0.17%, Vit. A, D_3, E, and K in oil 0.25%, B-complex Vitamins (except B_{12}) in starch 0.10%, and sufficient iodized calcium carbonate to supply 1 mg iodine per lb were fed for 2 weeks. For purposes of comparison, a group of chicks depleted of Vit. B_{12} was also fed a standard chick starter. Because the basal diet was deficient in lysine(1,2), certain groups were fed additional lysine in the form of DL-lysine monohydrochloride monohydrate to provide 0.2% L-lysine. Since the requirement for Vit. B_{12} on high protein diets is increased(3), sufficient APF supplement was added to provide for this deficiency (25 μg B_{12} per lb of ration). This amount supplied approximately 10 times more Vit. B_{12} than the standard chick starter mash used for comparison.

Results. Table I gives the gross effects of the various supplements at four weeks of age. During the experiment it was observed that the birds on the high sesame meal-Vit. B_{12} deficient diet (Group 2) had underdeveloped, pale combs, whereas the combs of chicks receiving the APF supplement with or without lysine (Groups 3 and 5) showed normal red color and size comparable to those receiving the standard chick starter (Group 1).

Since comb growth and color are influenced by testicular function, the testes were examined to determine the state of their development. Testes produced on the basal diet were subnormal in weight, whereas the ones produced by an addition of both lysine and Vit. B_{12} showed an increase in weight of 88% over those on the basal diet. The histological differences were even more remarkable. As expected, the testes from chicks on

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* Presented before the Rocky Mountain Section, Society for Experimental Biology and Medicine, Salt Lake City, Nov. '49; and the Midwest Feed Manufacturers' Association, Kansas City, Feb. '45.

† Colorado Agricultural Experiment Station Scientific Journal Series No. 336.

1. Grau, C. R., and Almquist, H. J., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 187.
2. Grau, C. R., *J. Nutrition,* 1948, v36, 99.
3. Hartman, A. M., Dryden, L. P., and Cary, C. A., Agric., Res. Admin. BDIM-INF-76, July 1949.

TABLE I. Effect on Comb, Testis, and Body Weight of Supplementing a Sesame Meal Diet with APF and with DL-Lysine Hydrochloride.

Group	Diet and supplement	Body wt 4 wk (g)	(Adjusted means)* Comb wt, mg	% diff. from control	(Adjusted means)* Testis wt, mg	% diff. from control
1	Chick starter control	311	1028.3	0	95.7	0
2	70% sesame meal basal	211	514.6	—50	65.1	—32
3	Basal + APF	245	1060.6	+ 3	109.2	+14
4	Basal + lysine	203	653.8	—36	72.4	—24
5	Basal + APF + lysine	213	828.7	—20	121.8	+27
Min. diff. required for significance						
at 5% level			312.7		21.9	
at 1% level			412.8		28.9	

* Means adjusted for body wt by covariance analysis.

the standard starter ration appeared normal (Fig. 1). In the testes produced on the sesame meal basal (Fig. 2) the seminiferous tubules were markedly decreased in number and size and they were separated by increased intertubular spaces. Leydig cells were fewer in number and many showed pyknotic nuclei. The condition resembled that of early hypophysectomy in the chick(4). Addition of Vit. B_{12} resulted in normal testicular weight due to enlarged tubules with epithelium of greater height, normal intertubular spaces and apparently normal, functional Leydig cells (Fig. 3). This response suggested an increased action of pituitary gonadotrophin, particularly of the type that stimulates growth of Leydig cells.

Lysine alone did not substantially change the weight of the testes. The histological appearance, however, indicates that supplementation with lysine actually had a detrimental effect as illustrated by the tubules filled with debris and the marked lymphocytic infiltration in intertubular spaces (Fig. 4). The Leydig cells were compressed in narrow intertubular spaces and many displayed the pyknotic nuclei previously mentioned as characteristic of hypophysectomy.

Supplementation with lysine and Vit. B_{12} produced slightly greater testicular growth and differentiation of the seminiferous epithelium (Fig. 5 and 6) than seen in the normal controls (Fig. 1). These birds showed testes with larger tubules, increased height of germinal epithelium and scattered mitotic figures. The intertubular spaces were enlarged and filled with an increased number of Leydig cells that appeared to be functional.

Discussion. The data obtained in this study suggest that Vit. B_{12} plays a role in the development and function of the reproductive organs. This view is supported by the work of several investigators. Hartman, Dryden and Cary(3) demonstrated a stimulatory effect of Vit. B_{12} on the reproductive organs of the female rat deficient in that vitamin. An analogous relationship was shown to exist by Hertz and Tullner(5) with respect to the need for pteroylglutamic acid to maintain sensitivity of the chick oviduct to estrogen, apparently by stimulating protein synthesis. Similarly, Vit. B_{12} seems to accelerate the utilization of amino acids for protein synthesis (6). The view that chicks deficient in Vit. B_{12} are unable to synthesize the necessary protein for gonadal response to gonadotrophin is further supported by the work of Chow(7) who demonstrated a lack of response to this hormone by Vit. B_{12}-deficient rats of both sexes.

Summary. Feeding cockerels depleted of

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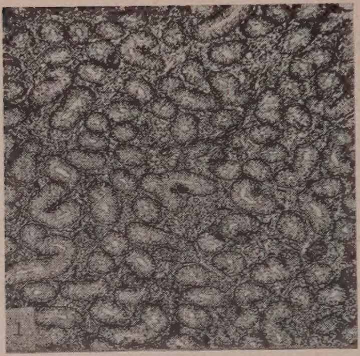
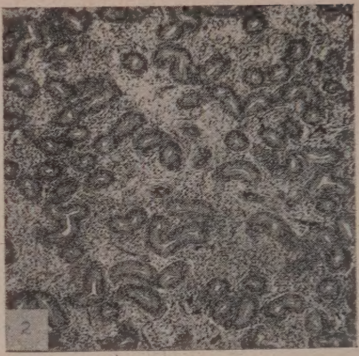
4. Koch, F. C., 1946, personal communication.

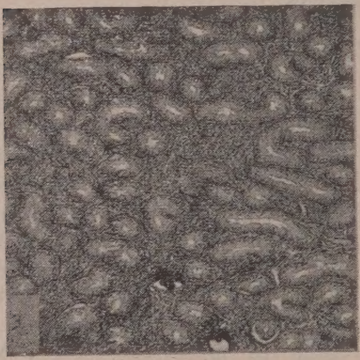
5. Hertz, Roy and Tullner, W. W., *Endocrinol.*, 1949, 278; *Science,* 1948, v107, 300; PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 113.

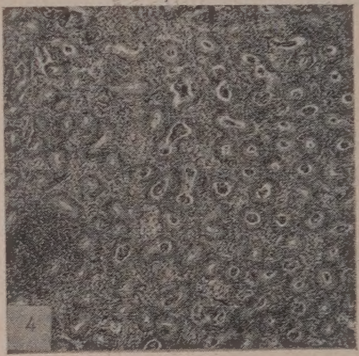
6. Charkey, L. W., Wilgus, H. S., Patton, A. R., and Gassner, F. X., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 21.

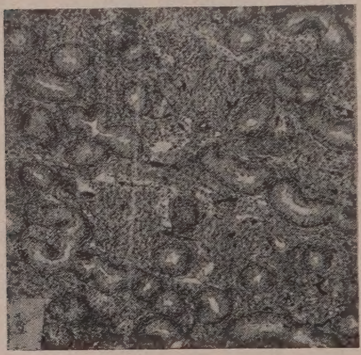
7. Chow, B. F., and Barrows, Lois. Abstract of Meetings, 12A, American Chemical Society, Philadelphia, April 1950.











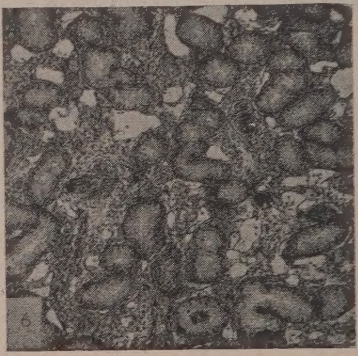


FIG. 1. Normal testes from chicks fed the control chick starter mash. 200×.

FIG. 2. Testes from chicks on deficient sesame meal basal diet. These show a decrease in diameter of tubules and an increase in the intertubular space, but with fewer Leydig cells in the intertubular spaces. 200×.

FIG. 3. Testis from a chick fed sesame meal basal diet and APF supplement. The number of tubules and the tubular diameter resembles strongly that seen in the control. The interstitial spaces are not excessive and have a normal number of Leydig cells. 200×.

FIG. 4. Testis from a chick fed sesame meal basal with lysine supplementation. This testis reflects histologically the failure of lysine to restore normal morphology of the testes. The tubules are of small diameter with low epithelium and have, in addition, intratubular debris. Also, the interstitial tissue, which has few Leydig cells, shows infiltration with lymphocytes. 200×.

FIG. 5 and 6. Two different areas of a testis from a chick fed the sesame meal basal diet supplemented with both lysine and APF. The striking increase in weight of the experimental testes over that of the control testes is borne out by the appearance of this gonad. The tubules are of greater diameter and show an increased epithelial thickness. There is an increase in the intertubular spaces which are filled with well-formed Leydig cells. 200×.

Vit. B_{12} from the second to fourth weeks of age on a 70% sesame meal diet deficient in Vit. B_{12} and lysine resulted in a failure of morphological differentiation of testicular tissue accompanied by a decrease in comb growth. Supplementation with Vit. B_{12} and lysine produced markedly greater testicular growth and differentiation over that in the normal controls.

The authors acknowledge with thanks gifts of APF Supplement No. 3 from Merck and Company, DL-lysine from E. I. duPont de Nemours and Co., Protamone from Cerophyl Laboratories, Inc., sodium proteinate from the Archer Daniels Midland Co., and sesame meal from Industrias Unides, Nuevo Laredo, Mexico.

Received July 31, 1950. P.S.E.B.M., 1950, v75.

Presence of Myelokentric and Lymphokentric Acid in Sera of Patients with Lymphomatoid Diseases. (18288)

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The urine of patients with leukemia and other lymphomatoid diseases has been shown to contain substances(1) that we have named myelokentric and lymphokentric acid(2). These substances seem to play a part in the proliferation and possibly the maturation of myeloid and lymphoid cells. These substances have been found also in the organs(3) of patients with these diseases and in normal beef liver(4).

Heinle and his co-workers(5) have shown that relatively small amounts of material extracted by methods other than ours from similar urinary sources showed considerable potency for proliferation of myeloid cells. From this work we have considered the possibility that small amounts* of plasma or serum from patients with these diseases might contain sufficient conjugated material to produce reactions in the organs of animals. Therefore, the sera from three types of leukemia, Hodgkin's disease and normal individuals have been given to guinea pigs, and at various intervals after injection the animals were sacrificed and the organs studied.

Material and methods. Under sterile precautions 20 to 40 ml of blood was removed from each subject, placed in a sterile tube, allowed to clot, and later centrifuged for ½ hour at 3000 r.p.m. For injection, 5 ml of the serum was drawn off and introduced into the peritoneal cavity of a guinea pig. Usually two animals were used, one of them being sacrificed after 24 hours, the other after 48 hours; in 2 experiments the interval was 8 hours. Sections were made of liver, spleen, lymph nodes, adrenal, kidney and bone marrow.

Many of the sera from normal individuals were collected at the time of donations of blood for transfusions. To obviate any discrepancy due to the alarm mechanism which might occur incidental to the removal of blood, samples in 3 instances were taken at the start of the donation as well as at the end. Samples of serum were obtained from 9 patients with chronic myeloid leukemia, 6 patients with chronic lymphoid leukemia, 6 patients with monocytic leukemia, 4 patients with Hodgkin's disease and 12 normal individuals. Table I shows the distribution of

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1. Miller, F. R. and Turner, D. L., *Am. J. Med. Sc.*, 1943, v206, 146.

2. Turner, D. L. and Miller, F. R., *J. Biol. Chem.*, 1945, v161, 91.

3. Erf, L. A., Turner, D. L., and Miller, F. R., *Blood*, 1946, v1, 379.

4. Turner, D. L. and Miller, F. R., PROC. SOC. EXP. BIOL. AND MED., 1943, v54, 177.

5. Hirschmann, H., Heinle, R. W., and Wearn, J. T., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 5.

* Urine dose = 500 ml to 7.1. Versus serum dose = 5 ml.

TABLE I.

	No. of animals		
	8 hr	24 hr	48 hr
Chronic myeloid	2	13	10
,, lymphoid	2	7	6
Monocytic leukemia		6	6
Hodgkin's disease		3	4
Normal individuals		18	17
Total	4	47	43

the animals receiving these sera.

Results. In this report only the changes that were found in the lymph nodes are described, although all the organs that were sectioned were surveyed. The survey was made to determine whether any consistent picture of myeloid, lymphoid or mixed reaction could be found, *i.e.*, reactions that would be similar to those described following the administration of urinary extract(1).

In the organ survey it was found that minimal specific reactions occurred in the organs, other than the lymph nodes, of animals that received serum from patients with each of the 4 diseases.† It was also found, however, that in a certain number of the animals that received serum from normal individuals any one of the 3 reactions was elicited with, however, a lack of response in the lymph nodes. The lymph nodes, and these were usually mesenteric or caecal nodes, although some tracheal nodes were examined, seemed to fall into 4 groups depending on which serum was given. A description of these nodes follows.

Lymph nodes. The lymph nodes from the animals that were sacrificed at 8 and 24 hours after receiving the serum from patients with chronic myeloid leukemia revealed similar findings. There was lymphocytic destruction, depletion of the number of lymphocytes, edema of the intercellular spaces, pleomorphism of the cellular structure, a slight infiltration of polymorphonuclear leukocytes, a loss of follicles, and an apparent increase in the underlying connective tissue. A few scattered eosinophiles were found in the nodes of all animals (Fig. 1, 2).

The nodes from the 10 animals that were sacrificed at 48 hours after injection exhibited a beginning return of normal lymphoid cells, less destruction of lymphocytes, less pleomorphism and less edema. There was more fibrosis than in the previous group. Although a few scattered eosinophiles could be found in nodes from each animal, a definite eosinophilia occurred in the nodes from 2 of them.

The animals that received the serum from patients with chronic lymphoid leukemia were divided into 3 groups: those that were sacrificed at 8 hours, at 24 hours, and 48 hours. The nodes from those sacrificed at 8 hours showed a slight increase in lymphopoiesis and little increase in eosinophiles. The nodes from the 7 animals sacrificed at 24 hours exhibited an increase in size of the lymphocytic collar around the follicles, and an increased number of follicles. The cells tended toward a uniform size and there was a definite spotty increase in the number of eosinophiles. The nodes were frequently compact and rather densely populated with cells in 4 to 6 animals, while in 2 there was some intercellular edema, but in none was there pleomorphism nor a polymorphonuclear infiltration (Fig. 3, 4).

The nodes from the 6 animals sacrificed at 48 hours still exhibited an increase in lymphopoiesis. Intercellular edema occurred in those from 4, an eosinophilia in those from 3, and slight fibrosis in those of 3.

An eosinophilia occurred in the lymph nodes of 11 of the total group of 15 animals.

Nineteen animals were given serum from patients with Hodgkin's disease or monocytic leukemia. Nine of these were sacrificed 24 hours after injection and the other 9 were sacrificed 48 hours after injection. In the nodes of all these animals there was some increase in lymphopoiesis and some pleomorphism and a few polymorphonuclear leukocytes. An increase in elongated fibroblasts was also frequently apparent (Fig. 5, 6). In the nodes from 13 of the animals an increase in eosinophiles was evident, the 6 in which

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† Sera from patients with monocytic leukemia and Hodgkin's disease give the same or the mixed reaction.

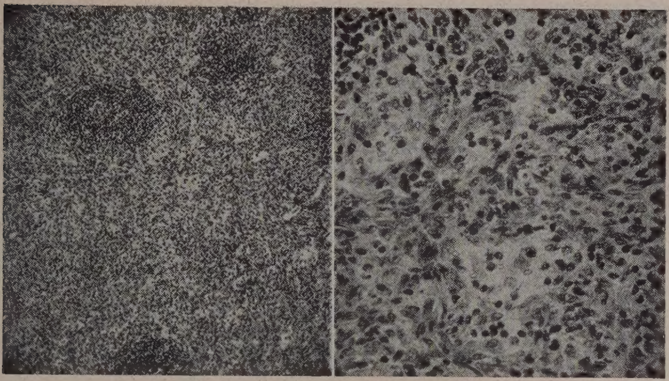

FIG. 1 and 2. ×50 and ×200. Lymph node from guinea pig 24 hr after inj. with 5 cc of serum from a patient with chronic myeloid leukemia.

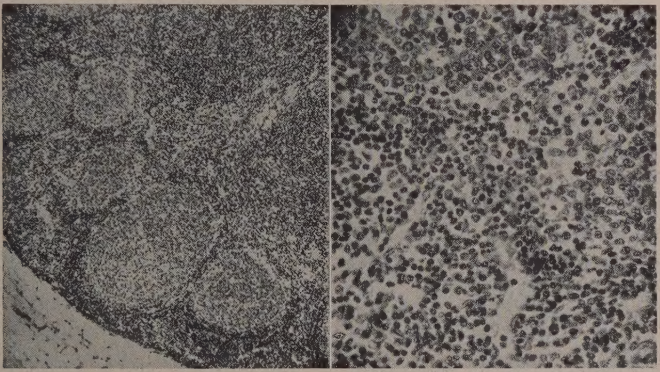

FIG. 3 and 4. ×50 and ×200. Lymph node from guinea pig 24 hr after inj. with 5 cc of serum from a patient with chronic lymphoid leukemia.

this did not occur were animals sacrificed at 48 hours.

Thirty-five animals were given serum from 12 normal individuals. Eighteen of these were sacrificed at 24 hours, and 17 at 48 hours after injection. The nodes from 29 of these animals did not show much change from those of normal animals. In the nodes from 5 animals there was a moderate eosinophilia and in 2 there was some intercellular edema, slight pleomorphism and some increase in lymphopoiesis. All nodes from all animals showed a few scattered eosinophils.

Summary and conclusions. The lymph nodes from animals that received serum from patients with chronic myeloid leukemia have a tendency to the same loss of lymphoid structure that is seen after the use of cortisone or ACTH; however, an increase in fibrosis and underlying connective tissue is also apparent. In these nodes, too, there is some slight increase in polymorphonuclear neutrophiles. The nodes from the 2 groups that received sera from patients with chronic lymphoid leukemia, Hodgkin's disease and monocytic leukemia all showed a tendency to increased lymphoid production, although the third group (Hodgkin's and monocytic leukemia) also showed some increase in fibrosis. In both these groups a definite increase in eosinophiles occurred. Probably the lack of increase in underlying connective tissue in the "lymphoid group" segregates it from the "myeloid and the Hodgkin's monocytic

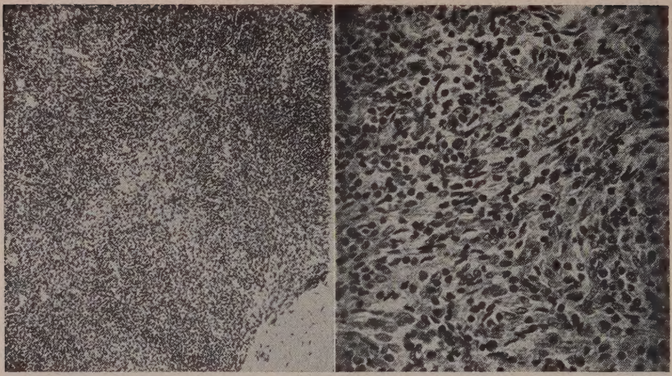

FIG. 5 and 6. ×50 and ×200. Lymph node from guinea pig 24 hr after inj. with 5 cc of serum from a patient with monocytic leukemia.

groups." The nodes from the animals that received sera from normal individuals showed on the whole nothing unusual.

The sera of patients with chronic myeloid leukemia bring about changes similar to those ascribed to cortisone itself or as initiated in action by ACTH in so far as the lymph node change is concerned. Myelokentric acid brings about similar lymph node changes and, as does cortisone, it also brings about increases in erythropoiesis, myelo-leukopoiesis and platelet formation. We do not, however, believe that it is cortisone.

The increased lymphopoiesis in the lymph nodes of animals that received serum from patients with chronic lymphoid leukemia is similar to that which we have described in animals that have received lymphokentric acid in urinary extracts.

It would seem then that at least 2 and possibly 3 pathologic pictures may be made out in the nodes of these animals. This would seem to indicate that there are substances in the blood of these patients that are probably conjugates of myelokentric and lymphokentric acid. The presence of these may be detected in the sera of patients with lymphomatoid disease by bioassay. It also strengthens our contention that these are humoral hormones.

This work was aided by a grant from the American Cancer Society.

We wish to thank Mrs. Diva de Andino for technical help.

Received August 2, 1950. P.S.E.B.M., 1950, v75.

Variations in Serum Cholesterol of Normal Rabbits after Injection of Hypercholesterolemic Rabbit Plasma. (18289)

ELLIOT OPPENHEIM AND MAURICE BRUGER.

From the Department of Medicine, New York University Post-Graduate Medical School, New York University-Bellevue Medical Center.

Investigators have long been interested in producing atherosclerosis by intravenous injection of cholesterol suspensions. Dewey(1), Fitz and Bruger(2), Bevans(3) and Pollak (4) have reported their experience with colloidal solutions of free cholesterol. The present experiments were carried out to de-

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1. Dewey, K., *Arch. Int. Med.*, 1916, v17, 757.
2. Fitz, F., and Bruger, M., *J. Biol. Chem.*, 1936, v114, xxxv.
3. Bevans, M., *Am. J. Med.*, 1949, v6, 103.
4. Pollak, O. J., *Am. Heart J.*, 1949, v38, 455.

TABLE I. Control Group.

Exp. No.	Experiment	Chol. fraction	Time									
			0	5 min.	15 min.	30 min.	60 min.	2 hr	4 hr	6 hr	24 hr	48 hr
1	20 ml normal rabbit plasma containing 13 mg cholesterol. Fasting 0 to 24 hr	T.	75	56	67	68	72	63	63	61	102	106
		E.	46	35	42	42	45	40	42	38	64	63
		F.	29	21	25	26	27	23	21	23	38	43
		E/F	1.6	1.7	1.7	1.6	1.7	1.7	2.0	1.7	1.7	1.5
2	20 ml normal rabbit plasma containing 7 mg cholesterol. Fasting 0 to 24 hr	T.	66	61	61	62	59	58	58	59	78	
		E.	44	40	41	43	41	40	40	40	50	
		F.	22	21	20	19	19	17	18	19	29	
		E/F	2.0	1.9	2.1	2.2	2.2	2.3	2.2	2.1	1.7	
3	20 ml normal rabbit plasma containing 5 mg cholesterol. Fasting 0 to 24 hr	T.	38	35	30	30	25	25	24	25	30	
		E.	27	22	22	22	18	17	16	17	19	
		F.	11	12	9	8	7	8	8	8	11	
		E/F	2.4	1.8	2.6	2.9	2.4	2.1	2.0	2.2	1.8	
4	20 ml normal saline. Fasting 0 to 24 hr	T.	66	60	61	56	52	90	87	88	100	64
		E.	43	37	39	35	32	57	56	56	56	41
		F.	23	24	22	21	21	33	30	32	45	22
		E/F	1.9	1.6	1.8	1.7	1.5	1.7	1.9	1.8	1.2	1.8
5	Nothing given. Non-fasting	T.	65	59	59	54	66	65	65	54	75	65
		E.	37	37	30	26	38	41	37	26	27	35
		F.	28	23	28	28	27	24	28	28	48	30
		E/F	1.3	1.6	1.1	0.9	1.4	1.7	1.3	0.9	0.6	1.2

T = Total cholesterol mg/100 ml.
E = Esterified cholesterol mg/100 ml.
F = Free cholesterol mg/100 ml.
E/F = Esterified cholesterol/free cholesterol.

termine the effect of the intravenous administration of hypercholesterolemic rabbit plasma on the free and total serum cholesterol of normal rabbits.

Procedure. Hypercholesterolemic plasma was obtained by cardiac puncture from male rabbits, 3 to 6 months old, which were fed 3 g of cholesterol per week for 8 weeks or longer. The recipient animals were male rabbits of the same age group which received no cholesterol in their diet. The control group consisted of 5 normal male rabbits. In 3, 20 ml of normal rabbit plasma containing an average of 8.3 mg of cholesterol was injected into an ear vein. In one rabbit, 20 ml normal saline was injected and in another, nothing was given by mouth or vein. Specimens of blood were obtained by cardiac puncture in the non-anesthetized state at the following intervals: before injection, 5, 15, 30 and 60 minutes, 2, 4, 6 and 24 hours and at times 48 hours after the injection. Duplicate determinations of the free and total cholesterol in the sera were made by the Schoenheimer-Sperry method(5). In the experimental group, each of 4 normal male rabbits was injected intravenously with 20 ml of plasma containing an average of 132 mg of cholesterol. Blood specimens were obtained and examined in the same manner as in the control group.

Results. Table I presents the data obtained in the control experiments. It will be noted that the injection of normal rabbit plasma containing a small amount of cholesterol had little effect on the total cholesterol or E/F ratio for 6 hours. In 24 hours, however, the total cholesterol increased significantly in 2 experiments (Exp. 1 and 2). The same results were obtained when 20 ml normal saline were injected into the ear vein or when nothing was given parenterally or by mouth. Table II presents the data obtained in the experimental studies. The total serum cholesterol increased rapidly after the injection, the highest values being obtained in the 5 minute specimen. Thereafter, the serum cholesterol fell gradually, the lowest value being obtained in 6 hours or less. In 24 hours, the total serum cholesterol was significantly increased above the 6 hour level as in the control group. The E/F ratio was

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5. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, v106, 745.

TABLE II. Experimental Group.

			Time									
Exp. No.	Experiment	Chol. fraction	0	5 min.	15 min.	30 min.	60 min.	2 hr	4 hr	6 hr	24 hr	48 hr
1	20 ml rabbit plasma containing 130 mg cholesterol. Non-fasting	T.	59	162	151	145	129	97	74	74	101	94
		E.	33	102	101	96	82	57	44	44	57	50
		F.	26	60	50	49	47	40	30	30	44	45
		E/F	1.3	1.7	2.0	2.0	1.8	1.4	1.4	1.5	1.3	1.1
2	20 ml rabbit plasma containing 133 mg cholesterol. Fasting 0 to 24 hr	T.	52	187	180	174	148	123	97	70	96	
		E.	28	118	117	110	86	76	54	35	51	
		F.	24	69	64	65	62	48	43	35	45	
		E/F	1.2	1.7	1.8	1.7	1.4	1.6	1.3	1.0	1.1	
3	20 ml rabbit plasma containing 130 mg cholesterol. Fasting 0 to 24 hr	T.	49	153	145	138	104	69	70	70	84	
		E.	35	105	102	98	72	45	48	46	54	
		F.	14	48	43	39	32	24	22	25	30	
		E/F	2.5	2.2	2.4	2.5	2.3	1.8	2.2	1.9	1.8	
4	20 ml rabbit plasma containing 135 mg cholesterol. Fasting 0 to 24 hr	T.	60	205	197	161	154	138	94	85	108	

not altered significantly.

Discussion. Bevans(3) noted a secondary rise in total serum cholesterol but not in free cholesterol in rabbits 24 hours after the intravenous injection of 0.5 g of a colloidal solution of free cholesterol. She interpreted this to be due to clearing of the injected cholesterol from the blood and a slow return after esterification. In the experimental group, here reported, an increase in the total serum cholesterol occurred in 24 hours. A secondary rise in the free cholesterol must have occurred simultaneously since there was no change in the E/F ratio. The variation of these results from those reported by Bevans may be explained by the fact that hypercholesterolemic plasma containing free and esterified cholesterol was injected in these experiments. In the control experiments, however (Table I), an increase in the total serum cholesterol was also noted in 24 hours after the injection of normal rabbit plasma, normal saline or when nothing was given. The rise in total serum cholesterol is therefore not an intrinsic response to the injection of cholesterol as reported by Bevans but apparently is related to the mechanics of performing the experiment, *i.e.*, the result of serial blood letting and/or trauma.

Summary. The intravenous administration of hypercholesterolemic rabbit plasma in normal rabbits resulted in a rapid increase in total serum cholesterol followed by a gradual decrease. The highest value was obtained in 5 minutes and in 6 hours or less the total serum cholesterol had reached the lowest post-injection level. In 24 hours, the total serum cholesterol was significantly increased above the 6 hour level with no significant alteration in the E/F ratio. Rabbits receiving normal rabbit plasma, normal saline or no injection showed little alteration in the total serum cholesterol for 6 hours. In 24 hours, the total serum cholesterol likewise increased significantly compared to the 6 hour level. The rise in total cholesterol at 24 hours is apparently related to the mechanics of performing the experiment rather than to the injection of cholesterol.

Received August 3, 1950. P.S.E.B.M., 1950, v75.

Failure of Adrenalectomy Immediately Following Stress to Prevent Eosinopenia in Rats.* (18290)

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It is an old clinical observation that eosinopenia frequently accompanies severe illness. Dalton and Selye described this as a constant part of the general alarm reaction(1). Subsequent work(2,3) has demonstrated that a fall in eosinophiles may be produced by administration of 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone in man. Since epinephrine, in the presence of intact pituitary and adrenal function is also capable of initiating this phenomenon, the change in circulating eosinophiles induced by epinephrine has become the basis for clinical tests of adrenal cortical responsiveness(4).

After epinephrine is injected into normal animals, the number of eosinophiles in the peripheral blood decreases gradually during a 4 hour period. It has not been determined whether the progressive decrease is due to the action of adrenal steroids secreted within a few minutes after the epinephrine stimulation, or indicates that there must necessarily be a continued elaboration of adrenal hormones during the time required for development of the maximum eosinopenia. The experiments described in this report were designed to answer this question. Bilateral adrenalectomies were performed rapidly on a series of rats so that both adrenal pedicles were clamped within 10 or 15 minutes after epinephrine administration. Eosinopenia was as great in these as in normal or in sham-operated animals. It is evident, therefore, that the quantity of adrenal steroids secreted within 10 to 15 minutes after epinephrine had been given was sufficient to induce eosinopenia. In addition, the fall in circulating eosinophiles was gradual and progressive over 4 hours in each group of animals, indicating that a time interval is necessary before the effect of the steroids on eosinophiles is apparent.

Method. Untrained, albino rats of the Fairfield strain, males and females, weighing 125 to 250 g were used. The test consisted of picking up an animal and injecting it subcutaneously with 0.2 mg/kg of epinephrine,† after which it was restrained in a tail cage for the 4 hours of the procedure. Counts of the total eosinophiles were made approximately 10 minutes after the injection, at 2 hours, and at 4 hours after the initial alarm of handling. Eosinophile counts were done by a modification of the Randolph technic(5), counting a total of 11.4 cu mm of a 1/20 dilution of tail blood (0.57 cu mm of blood) in aqueous 50% propylene glycol with 0.1% eosin. The standard deviation measured by 18 counts with a mean value of 131 eosinophiles per cu mm was 9 eosinophiles per cu mm. Single stage adrenalectomies were performed rapidly under ether anesthesia through a single mid-line dorsal skin incision, under direct visualization, with minimal traction on the adrenal glands themselves. Sham-adrenalectomy was done in the same manner except that after opening the abdomen through an identical incision the only procedure was displacement of the intestines and gentle traction on the perirenal fat. The time required to complete an adrenalectomy was measured from the time an animal was first picked up until the pedicle of the second gland was clamped. With practice, it became possible

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* This work was supported in part by a grant from the Lederle Laboratories Division of the American Cyanamid Co.

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2. Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, v3, 755.

3. Perara, G. A., Pines, K. L., Hamilton, H. B., and Vislocky, K., *Am. J. Med.*, 1949, v7, 56.

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† Parke-Davis Adrenalin, diluted 1/5 with distilled water and prepared immediately before use.

5. Randolph, T. G., *J. Allergy*, 1944, v15, 89.

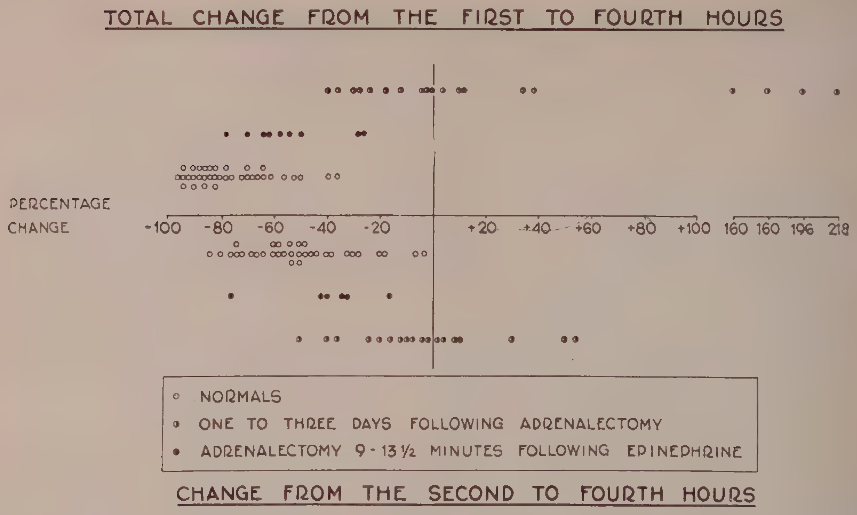

Fig. 1.

Changes in eosinophile counts in rats following injection of 0.2 mg/kg epinephrine subcutaneously.

to remove a rat from its cage, inject the epinephrine and complete the adrenalectomy rapidly enough so that the first count could be taken from ten to fifteen minutes after the animal had first been handled.

Results. The decrease in eosinophiles which had occurred by the end of 4 hours in response to epinephrine in 35 normal rats had a median value of 80% (range 38% to 95%). Three tests during sham-adrenalectomy gave results similar to the normals. The latter animals again responded normally 24 hours after the operation. Nineteen tests were done from one to 3 days after adrenalectomy as a test of the completeness of the operation and in order to make sure that epinephrine stimulation would no longer cause an eosinopenia. The median value for percentage change in eosinophiles showed no fall (range 40% fall to 220% rise).

In the 9 rats given epinephrine and adrenalectomized within approximately 10 minutes (13½, 11, 10, 10, 10, 10, 10, 9 and 9 minutes), the median value of the fall in eosinophiles was 58% (range 78% to 26%).

In order to compare the groups for differences in the progressive and gradual nature of the fall in eosinophiles, the changes in circulating eosinophiles during the second half of the 4 hour period were determined. The observed values for the final eosinophile levels relative to the level at the second hour were: normals, a median fall of 50% (range 0% to 80%); adrenalectomized animals, a median fall of 5% (range 60% rise to 60% fall); and in rats with adrenalectomy 9 to 13½ minutes following epinephrine injection, a median fall of 38% (range 15% to 78%). The results of all tests are summarized in Fig. 1.

Discussion. These results confirm the observation of Recant *et al.*(4) that eosinopenia follows injection of epinephrine in intact, but not in adrenalectomized rats. A significant decrease in circulating eosinophiles occurs even if the adrenals are removed ten minutes after epinephrine injection. Therefore, an appreciable quantity of adrenal steroids must be secreted within 10 minutes of the initial alarm, under the conditions of these experiments. This conclusion is in agreement with the observation of Long(6) that there is a significant fall in adrenal ascorbic acid within 20 minutes after stress in rats. In addition, Vogt(7) has shown that

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6. Long, C. N. H., *Bull. N. Y. Acad. Med.*, 1947, v23, 260.

the cortical hormone content of the blood of the adrenal veins of dogs rises within 15 minutes after an infusion of physiological amounts of epinephrine. Despite the prevention of further cortical hormone production by adrenalectomy approximately 10 minutes after epinephrine injection, the continued normal 4 hour fall in circulating eosinophiles takes place. This indicates that the progressive eosinopenia occurring in normal rats need not depend on persistent or increasing elaboration and secretion of adrenal hormones.

Conclusions. 1. In rats, an eosinopenia follows stress even though adrenalectomy is completed within 10 minutes. 2. The progressive fall in circulating eosinophiles over a 4 hour period occurs even without the continued secretion of the adrenal cortex.

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Received August 7, 1950. P.S.E.B.M., 1950, v75.

Effect of Sinusoidal Stimulation upon Circulation Time.* (18291)

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The primary motivation for this work was the hope of Dr. V. L. Tichy, attending in Surgery, Crile V. A. Hospital, to cut down on the incidence of thrombosis and embolism, post-operatively. With this end in view, Dr. Tichy approached the author late in 1946 with the desire to stimulate electrically, with periodic stimulation, the calf muscles of post-operative and other patients confined to bed. Sinusoidal stimulation was suggested by the author as a likely possibility. A routine technic was then established for post-operative sinusoidal stimulation of the calves of both legs. A preliminary report of this work was given by Dr. Tichy and the author before the Cleveland Chapter of the American Society of Experimental Biology and Medicine in November, 1947. Since then two clinical reports have been given(1,2).

The present report deals with the investigative results on the effect of sinusoidal stimulation (such as in being used post-operatively) on circulation time. The effects of sinusoidal stimulation upon circulation time have not previously been reported. However, Gaskell(3) and Bulbring and Burn(4) more recently, studied the blood flow during muscular contraction produced by faradic stimulation. As regards the effect of exercise, Smith, Allen, and Craig(5) have shown that lifting and dropping a leg, 40 times per minute, while wiggling the foot vigorously at the same time, reduced the circulation time in 4 patients by 24% to 40%.

Blood chemical changes. In order to ascertain the effect of sinusoidal stimulation upon circulation time, two types of examinations have been made. The first was suggested by Dr. J. R. Kahn, Director of Laboratories, Crile V. A. Hospital, and was performed by Leonard Skaggs, chemist at this hospital. Venous blood was drawn from the foot of stimulated as well as non-stimulated patients 24 hours post-operatively, and studies were made of the O_2 and CO_2 tensions, and CO_2 combining power of the specimens. These studies showed that sinusoidal stimulation has no definite effect on blood O_2, plasma CO_2, or the plasma CO_2 combining power.

Sodium cyanide test. In searching for a simple, accurate, yet comparatively safe method, the use of the sodium cyanide test as developed by Smith, Allen, and Craig(5) seemed the most feasible. This method is objective because the end point consists of a gasp resulting from stimulation of the carotid sinus by sodium cyanide. Two percent freshly prepared sodium cyanide was injected into the dorsal vein of the foot anterior to the medial malleolus. The dose was 0.2 to 0.22 mg per kg of body weight. Two types of technics were used. The first consisted in rendering the vein prominent by the usual methods, inserting the needle into the vein, waiting 30 seconds, then injecting the solution as rapidly as possible, after which the needle was removed, and the whole procedure repeated as often as necessary. It was soon discovered however that foot veins are not as easy to get into as the antecubital vein of the forearm. Very often several attempts had to be made before entering the vein. It seemed to the author that if the patient could be spared this repeated needling, and if the needle could be inserted once and maintained in the same position for the duration of the test, the factors of trauma and compression could be eliminated even better than by the 30 second waiting period. Hence the second technic was adopted for most of the tests. This consisted in finding the vein as before, inserting a needle, and connecting it to a 5% saline solution† which was allowed to drip into the vein. We thus had a ready place for injecting our solution whenever we wished. The objection that the solution itself promoted blood flow is overcome by performing the test at variable intervals before, during, and after stimulation. Having inserted a needle (23 gauge)† into the vein and connected it to a solution of 5% sodium chloride in water, we allowed the saline to drip into the vein very slowly. After a variable interval, the syringe was removed from the needle, the sodium cyanide injected and the reaction timed with a stop watch. As soon as the sodium cyanide injection was completed, the saline was again allowed to flow at the same slow rate. After another variable rest period, the calf of each leg was gently stimulated by placing under it an electrode connected to the sinusoidal current (22-24 stimulations per minute). The rest period before stimulation varied from one to 40 minutes. The actual time of stimulation varied between 9 and 30 minutes, after which, and while the calves were still stimulated, the circulation time test was repeated, using the same dose. The stimulation was then stopped but the drip resumed, and a final test was done after an interval of 15 to 30 minutes.

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* Published by permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the author.

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5. Smith, Lucian A., Allen, Edgar V., and Craig, W. McK., *Arch. Surg.*, 1940, v41, 1366.

Table I shows the results of 24 tests on 9 patients. Patient 9 had a subjective, therefore questionable, endpoint, namely, a precordial pressure. All others had definite visible gasps. Where a blank was obtained, as in case 3, column G, and case 4, column J, the test was repeated after a suitable interval. Case 7, column J has 2 end points, 44 and 47 seconds. I feel that we ought to disregard the first because it was subjective, even though the patient was very definite that he recognized an odor or taste of sulfur 3 seconds before having to catch his breath.

Because of the questionable safety of sodium cyanide, an attempt was made to repeat these tests using 20% sodium dehydrocholate (Endo Products). This drug, when injected in upper extremity veins, produces a sharp end point. However, repeated attempts to test circulation time by injection into the veins of the foot were entirely unsatisfactory. In the first place, blanks (absent end points) occur too frequently. Furthermore, the amount of drug necessary (6 to 10 cc) is apt to produce chemical thrombosis. Hence this drug has been discarded for lower extremity circulation time testing.

The ether in saline circulation time test as developed by Hitzig(6) was found much more satisfactory than sodium cyanide or sodium dehydrocholate, and is the present method of choice. Tests with ether and saline confirm the above results with regularity. Hence, it can be concluded that sinusoidal stimulation, sufficient to produce a mild muscular contraction, causes a reduction in circulation time.

Time control. Soon after instituting sinusoidal stimulation, post-operatively, it became

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† Normal saline is being used now, and 20 gauge needles.

6. Hitzig, W. M., *Am. Heart J.*, 1935, v10, 1080.

TABLE I. Effect of Sinusoidal Stimulation on Circulation Time.
Drug, sodium cyanide 2%; dose, 0.2 to 0.22 mg/kg body wt; end point, deep gasp; site, dorsum of foot (saphenous vein); current, sinusoidal, 60 cycles/sec.; strength, enough for mild contraction; No. of contraction, 24/min.; site of stimulation, each calf.

Pt. No.	Diagnosis	Wt, lbs.	Dose, cc	Method	Int. min.	CT, sec.	Int. min.	Stim. min.	CT, sec.	Int. min.	CT, sec.	Remarks
1	Herniorrhaphy	160	.72	Drip	5	32	1	15	22	30	33	Very definite end point
2	Fissure in ano, 22 hr post op.	137	.6	”	5	45	25	30	37	15	47	Test done while under stimulation
3	Renal stone, no operation	130	.6	”	85	B						Definite end point
					102	37	40	15	25	20	32	
4	Renal stone, no operation	160	.73	”	30	56	5	15	B			Definite end point
								30	46	20	56	
5	Subphrenic abscess, 1 mo. post oper.	120	.55	”	45	32	5	10	22			Needle fell out after J.
6	Ischio-rectal abscess	140	.65	”	50	80	5	25	35	10	B	Precordial pain.
										25	48	
7	CNS lues	155	.7	”	60	56	1	10	44 47			Patient noted sulfur odor before gasp. Needle out after J.
8	Hysteria	135	.6	Dripless		N						B. with 0.6 cc. Marked end point with 0.67 cc
			.67			64	1	9	44			
9	Multiple sclerosis	145	.7	Dripless		N						B. with 0.7 cc. Good endpoint with 0.75 cc.
			.75			34	15	10	22			

Code: Int.—Interval. CT—Circulation time. B—(blank) = No end point.

apparent that to take care of as few as 3 machines, there were required the full-time services of an attendant, day and night, 3 shifts, 7 days a week, or 4 attendants in all. Because of the shortage of qualified personnel, and to eliminate, if possible, the human factor of time control, it was felt that an automatic time control unit that would throw the current on and off at predetermined intervals might be a valuable adjunct in these cases. A machine was made to meet this need, (Teca Corp., N. Y. City). The principle is the use of a clock which instead of having one hour hand and one minute hand, has 2 minute hands, one of which is used to start the current, the other to interrupt it. It is thus possible to arrange any combination of time, within an hour, such as: 55 minutes-on, 5 minutes-off; 30-on, and 30-off; 40-on, and 20-off, etc. There are 2 pilot lights. One is ‘on’ at all times as long as the machine is connected to the house current. The other lights only with each stimulation.

To prevent a sudden stimulation after a period of rest, 2 thermistors are inserted into the circuit. These are resistors which permit a slowly increasing current to pass as they are heated to a maximum, thus allowing for a slow rise of current to the patient at the beginning of each cycle of treatment. It takes between 3 and 4 minutes for the current to reach its maximum and about 5 minutes for the thermistors to cool off.

Summary and conclusions. 1. Experimental evidence is presented that mild sinusoidal stimulation decreases circulation time. 2. A new sinusoidal stimulator is introduced, using the principle of automatic time control at predetermined intervals of stimulation alternating with periods of rest during units of one hour, and allowing a slow rise in current at the beginning of each cycle.

I wish to express my appreciation to all those already mentioned, and, in addition, to Mr. W. O. Hayes, Director of Pharmacy at Crile Veterans Administration Hospital, for their cooperation in this work, and to Miss Lois Perrine, Chief, Physical Therapy, for her technical assistance.

Received August 14, 1950. P.S.E.B.M., 1950, v75.

Salt Inhibition and Enhancement of Cytochrome, Succinic, and Dopa Oxidase Systems of Mouse Melanomas. (18292)

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Sodium chloride by itself as physiological saline, or in conjunction with other salts in balanced isotonic solutions has often been used in the preparation of various tissue extracts for enzyme and other studies and has generally been considered to be an essential or desirable constituent of physiological media. While this is undoubtedly valid and important for many purposes where cellular integrity or morphological criteria are of importance, the use of these solutions has been extended to general applications in the study and manipulation of enzyme systems whose optimal activity requirements may be quite different.

Since the effects of various ions have been reported for certain systems(1-6) the present studies were undertaken to determine the quantitative influence of graded concentrations of salts frequently employed for preparing tissue extracts, and of those currently used in this laboratory for the control of adsorption and elution of cytoplasmic particulates in chromatography(7).

Experimental procedures and results. Unless otherwise indicated, all experiments were done under the following general conditions: Tumors of the Harding-Passey or Cloudman S91 mouse melanoma strains were ground with Celite and distilled water to yield a 20% suspension, and the enzyme-carrying cytoplasmic constituents were separated from the nuclei and tissue debris by the short Celite column procedure(8). The various enzyme determinations were carried out manometrically at 38°C employing 0.5 or 0.8 ml of tissue extract, 0.2 ml of substrate and cofactors, 0.1 ml of 2 N KOH in the well, and 0.5 or 1.0 ml of salt solution of proper concentration to establish the molarity in the vessel indicated by the tables and figures.‡ Sodium succinate concentration in most experiments was kept at 0.014 molarity to minimize its own salt effect. The small amount of residual salt contributed by the tissue is ignored in these experiments. Relative enzymic activities were calculated from the first 30 minutes of uptake following the tipping of substrates. This period is essentially linear and similar results can be obtained by calculating from the first 10 minutes of oxygen consumption.

Effect of sodium chloride. The influence of a wide range of salt concentration on the succinoxidase, cytochrome oxidase, and dopa oxidase systems was tested from 1.0 molar through 0.0001 molar and terminating with distilled water.§ Fig. 1 shows the inhibition and enhancement of the 3 enzyme systems at the concentrations studied. Inhibition of succinoxidase was obtained at all concentra-

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* With the aid of Carson Brooks. N determinations made in the NIH micro-chemical laboratory under the supervision of William C. Alford.

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† Eight mg of dry cytochrome C (Nutritional Biochemical Corp.) dissolved in 4 ml of 0.5 M or 0.14 M sodium succinate to give vessel concentrations of 1.25×10^{-5} M cytochrome C, and 0.05 or 0.014 M sodium succinate. No buffer was added in the melanoma experiments where the effects of single salts were determined. The buffer capacity of the tissue and the relative neutrality of the salts employed kept the pH at approximately 7.

‡ No significant differences in response to salt have been observed between the two melanoma strains under the conditions studied.

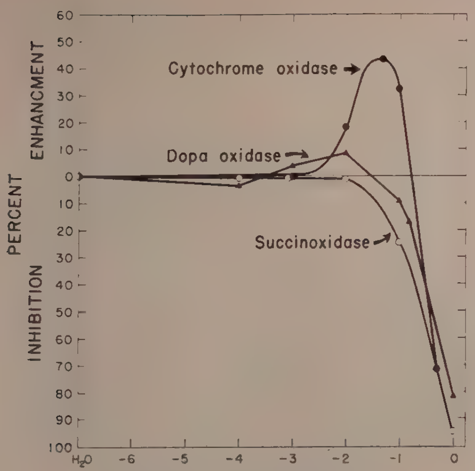

Fig. 1.

Relative enzymic inhibition and enhancement of mouse melanoma extracts at various NaCl concentrations.

tions above 0.01 molar. In no case was enhancement of succinoxidase activity noted at any concentration tested. When PPDA was substituted for succinate, as a measure of cytochrome oxidase activity, a distinct enhancement peak was obtained in the range between 0.01 and 0.1 molarity (Fig. 1). The dopa oxidase activity curve (Fig. 1) is similar to that obtained for succinoxidase. The slight enhancement obtained in the vicinity of 0.01 molar is not statistically significant, although a small enhancement effect at this concentration is not ruled out. Fig. 2 illustrates the degree of inhibition of the succinoxidase system by NaCl as compared with KCl from 0 to 0.7 molarity.‖

Inhibition by potassium chloride. Since sodium and potassium are antagonistic under some conditions(9) it was thought that the addition of the potassium ion might possibly counteract the toxicity of the sodium if the phenomena were due to cation inhibition. Table I indicates that no antagonistic effects were obtained with the concentrations used and that KCl acted in an additive way to increase the inhibition as though the Cl ion were the toxic agent; or alternately, that Na and K were behaving as a single toxic ion. An additional test was made, comparing distilled water with an isotonic solution containing a potassium-sodium ratio of 9 to 1. Similar inhibition effects were obtained with this solution. Fig. 2 illustrates the inhibition curve for KCl on the succinoxidase system.

Inhibition by potassium nitrate. Identification of chloride as an inhibitor for succinic dehydrogenase has been reported but with no quantitative data given(3). Since chloride was the common ion in the preceding experiments it appeared that it may have been responsible for the inhibition observed. In an attempt to check this, a similar experiment was done but with the substitution of NO_3 for the Cl anion. Fig. 3 indicates that es-

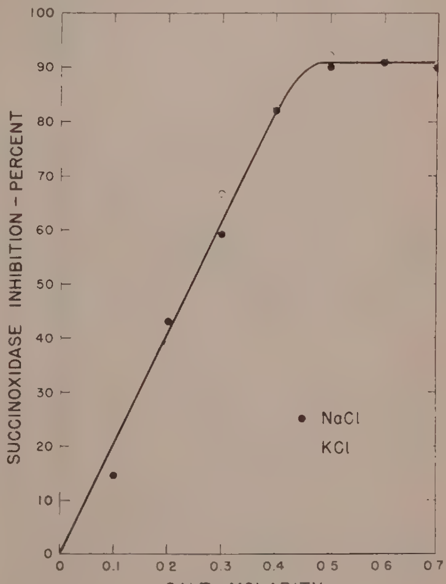

Fig. 2.

Inhibition of the succinoxidase system with sodium and potassium chloride.

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§ For purposes of orientation in estimating the absolute activity of the preparations in Fig. 1 the mean oxygen consumption of the various distilled water reference points were as follows: The succinoxidase mean of 10 vessels was 126, the cytochrome oxidase mean of 3 vessels was 190, and the dopa oxidase mean of 3 vessels was 59 μl O_2/hr.

‖ Oxygen consumption at the distilled water reference point was 126 μl O_2/hr. Each point represents the mean of 5 exps.

9. Rubinstein, D. L., *Biochem. Zeitschr.*, 1927, v182, 50.

TABLE I. Additive Inhibitory Effect of KCl When Superimposed on a Physiological NaCl Melanoma (Harding-Passey) Tissue Extract.

Salt combinations	Succinoxidase inhibition,† %
Distilled water suspension	0
0.9% NaCl suspension	47
0.9% NaCl suspension + .0001 M KCl	49
0.9% NaCl suspension + .001 M KCl	52
0.9% NaCl suspension + .01 M KCl	54
0.9% NaCl suspension + .1 M KCl	73
0.9% NaCl suspension + 1.0 M KCl	97
Distilled water extract	0
9 to 1 K-Na solution*	44
0.9% NaCl solution	49

* KCl, 8.946 g; NaCl, 0.76 g per l.

† The μl O_2/hr at 0 inhibition was 157 and 176 in the distilled water fractions.

sentially the same result was obtained with KNO_3 for succinoxidase as with KCl, but with the interesting difference that the dopa oxidase system was but slightly, if at all, inhibited.¶ These results demonstrate an interesting difference in enzyme-ion response between the two systems, although it did not establish the specific ion responsible for the inhibition of succinoxidase.

Inhibition and purification. Since this work was done with partially purified preparations it was thought that the inhibition might not be demonstrable in the cruder tissue extracts where there was a higher concentration of protective tissue colloids and tissue ions. To test this, an experiment was run in which the inhibition effect was tested at different stages of purification. As shown in Table II, there tends to be a slight contrary effect, but with no significant difference in the inhibition of the crude preparations as compared with more purified fractions.

Sodium chloride inhibition of tissue slices. The preceding results were obtained with cytoplasmic extracts or isolated intracellular granules. This posed the question as to whether the effect would be found when measuring the respiration of intact cells, since it has been reported that some cell membranes are impermeable to Na or Cl ions. Thin slices from a young, rapidly growing, mouse melanoma (Harding-Passey) were weighed and nearly equal amounts were transferred to chilled Warburg vessels. Two manometric series were run at 38°C employing succinate or dopa.** Table III indicates that similar inhibition effects were obtained with tissue slices as with the cytoplasmic extract or the isolated granules. The inhibiting effect of salt on the respiration of tissue slices suggests several possibilities. If the inhibition is due to the Cl ion it means that this anion can gain entrance to the cell interior rapidly and in substantial quantities, contrary to some reports(10), or acts indirectly in some manner, possibly affecting cell permeability. The similarity of the inhibition curve of granules and slices however, suggests the more direct inhibition of the intracellular succinoxidase system.

Reversibility of salt inhibition. A 20% Harding-Passey extract was prepared(8) in distilled water and the enzyme-carrying mel-

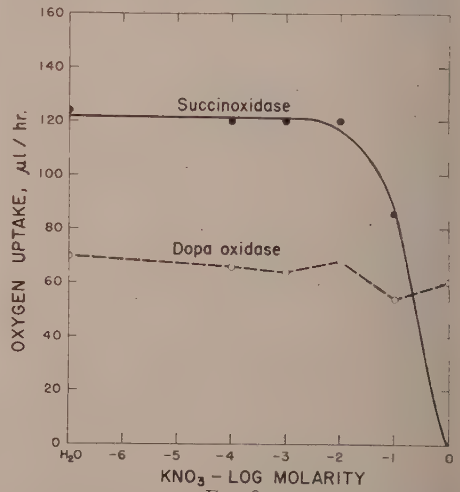

FIG. 3.

Differential inhibition of succinoxidase and dopa oxidase with KNO_3. (Cloudman S91 melanoma).

¶ Barring the possibility of an antagonistic effect, it would appear that the chloride anion is inhibitory for dopa oxidase while potassium and NO_3 are relatively non-toxic.

** Dopa oxidase was determined as described for succinoxidase except that 0.2 ml of dopa (1 mg) was employed as substrate.

10. Gersh, I., *Physiological Rev.*, 1941, v21, 242.

TABLE II. Effect of Purification on Salt Inhibition of the Succinoxidase System.

Fraction	Succinoxidase activity§ Dist. water $\mu l\ O_2/hr$	Succinoxidase activity§ Dist. water $Q_{O_2}(N)$	Succinoxidase activity§ 0.9% NaCl $\mu l\ O_2/hr$	Succinoxidase activity§ 0.9% NaCl $Q_{O_2}(N)$	Salt inhibition, %
Crude tumor homogenate*	426	122	261	75	39
Centrifugally cleared homogenate†	365	181	230	114	37
Granules centrifugally purified‡	134	244	89	162	34

* Harding-Passey mouse melanoma ground in mortar with distilled water to yield a 20% homogenate. Suspension contains intact cells, nuclei, and all cytoplasmic constituents.

† Crude homogenate centrifugally cleared to remove the bulk of the tissue debris, intact cells, and nuclei. The resulting supernatant contains most of the cytoplasmic elements.

‡ The cytoplasmic melanized granules partially purified by 2 cycle centrifugation. All the granules in the starting material were not recovered.

§ Determined manometrically with the addition of sodium succinate and cytochrome C. The tissue fraction volume was 1.0 ml. $Q_{O_2}(N) = \mu l\ O_2/hr/mg\ N$.

TABLE III. Inhibition of Succinoxidase in Harding-Passey Tumor Tissue Slices with Various Concentrations of Sodium Chloride.

NaCl conc. in vessel (molarity)	Inhibition, %
.0*	0†
.1	37
.2	49
.3	66
.4	83
.5	86
.6	89

* Distilled water.

† Oxygen consumption for the distilled water fraction was 70 $\mu l\ O_2/hr$ for 20 mg of tissue wet weight.

TABLE IV. Reversibility of Inhibition of the Dopa and Succinoxidase Systems Following Exposure to NaCl in the Cold or at 38°C.

Fraction	Succinoxidase activity* 4°C, %	Succinoxidase activity* 38°C, %	Dopa oxidase activity, 4°C, %
Granules maintained in dist. water	100	100	100
Granules maintained in 0.5 M NaCl	31	46	41
Salt replaced with dist. water	90	194	114

* The different temperature levels were run in separate experiments. The 4°C experiment employed Harding-Passey tissue, and the 38°C experiment was with the Cloudman S91 melanoma tissue. The $\mu l\ O_2/hr/ml$ for 4°C and 38°C with succinate was 67 and 63, and for dopa oxidase at 4°C was 108.

anized granules were centrifugally sedimented.[††] The resulting pellets were resuspended in 2 groups employing distilled water or 0.5 M NaCl as suspending fluids. After approximately 30 minutes at 4°C these suspensions were sedimented as before and again resuspended in fresh fluid but with one of the saline pellets taken up in distilled water rather than 0.5 M salt. A similar experiment was run but with the salt exposure carried out at 38°C. The reversibility of the effect of temporary exposure to strong salt, under the given conditions, is demonstrated by Table IV. The apparent enhancement in the second experiment of the fraction with the salt replaced by distilled water is probably due to the washing out of co-factors in the distilled water fraction at the higher temperature.

NaCl inhibition of liver mitochondria. While the salt effects reported here have been largely carried out on tumor tissue it is reasonably expected that similar findings for these enzyme systems will be found in other tissues. The following data show the results obtained with isolated liver mitochondria purified by differential centrifugation, as well as the response of relatively crude extract (Table V).

Discussion. The data of this report indicate that optimum activity of some of the oxidative enzymes carried on intracellular particles[‡‡] is not obtained with the conventional "physiological" concentrations of salts originally designed to meet the osmotic or other requirements of intact cells or mitochondrial elements. Thus, what may be the

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[††] Servall angle centrifuge, type SS-1, 9″ rotor, sedimented at approximately 10,000 times gravity for 15 min. plus a run up to 18,000 x g to pack the pellets.

TABLE V. Inhibition of Normal Mouse Liver Preparations with Sodium Chloride at Various Levels of Activity and Purification.

Fractions	Succinoxidase activity µl O_2/hr/ml	Q_{O_2} (N)	% inhibition
Crude extract*			
Distilled water	1092	284	4
Distilled water + .0001 M NaCl	1140	297	0
Distilled water + .001 M NaCl	1104	288	3
Distilled water + .01 M NaCl	1128	294	1
Distilled water + .1 M NaCl	768	200	33
Distilled water + 1.0 M NaCl	96	25	92
Isolated mitochondria†			
Distilled water	1880	3123	0
0.1 M NaCl	1048	1741	44

* Prepared by the short column procedure(8) in 10% sucrose and run manometrically under conditions of ionic substrates and co-factors for submaximal succinoxidase activity.

† Purified by differential centrifugation and run manometrically under conditions(6) for maximum activity.

best ionic conditions for maintaining morphology are not optimum for demonstrating maximum enzyme activity.

In determining enzyme rates, the highest activities obtained in the manometric vessel may not be the normal biological rates at the site of function in the cell(13). Thus, if in nature the succinoxidase system is exposed to "physiological" concentrations of sodium and potassium salts the present data indicate that its normal operative rate may be materially lower than the optimum potential rate. Some degree of normal enzyme inhibition would be advantageous considering the operative flexibility it would provide the system for acceleration and depression of the metabolism of the cell. These data thus emphasize the possible role that ions may play in the automatic regulation of enzymic rates as a result of ion movements or shifts across cell membranes.

The data establish that sodium and potassium chloride are distinctly inhibitory for certain enzyme systems. These effects, however, may be outweighed in some studies where salts are used to preserve the integrity of labile sub-cellular elements during isolation and purification procedures. The data, however, also indicate that salts may be employed in the preparation of material if they are removed or carefully corrected for in the measurement of enzyme activity. It should be noted that many of the values reported in the literature for these enzymes have been measured under various degrees of ionic inhibition and a correction factor should be applied when comparing them with optimum values.

It may be emphasized, that the inhibiting and enhancing results reported here have been primarily obtained with single salts under *in vitro* conditions. The synergistic and antagonistic effects of salt combinations on the various enzyme systems are yet to be systematically explored.

Summary. The quantitative inhibiting effects of NaCl, KCl, and KNO_3 on the succinoxidase, cytochrome oxidase, and dopa oxidase systems are reported. Enhancement, exceeding 40%, of the cytochrome system at certain concentrations of NaCl was observed. No significant enhancement of the succinoxidase or the dopa oxidase systems with the above salts was found at any of the concentrations tested. Inhibition of the succinoxidase system with NaCl and KCl was a rapidly increasing function from about 0.01 through 1.0 molarity, with physiological concentrations (0.9%) inhibiting up to about 50%. Reversibility of this effect was shown. No significant difference in degree of inhibition was found between crude and purified preparations. A differential inhibition response between the succinoxidase and dopa oxidase systems was observed for KNO_3. The former was inhibited as with KCl while dopa oxidase was inhibited but slightly if at all.

Received August 18, 1950. P.S.E.B.M., 1950, v75.

‡‡ Evidence has been presented that these melanized granules are mitochondrial elements(11,12).

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13. Bradfield, J. R. G., *Biological Reviews,* 1950, v25, 1.

Effect of Aureomycin, Chloramphenicol and Para-Aminobenzoic Acid on Myxoma and Vaccinia Viruses in the Egg.* (18293)

C. T. Lee.† (Introduced by M. H. D. Smith.)

Aureomycin and chloramphenicol (Chloromycetin ®) have proved effective in the treatment of both experimental and clinical infections caused by rickettsiae and viruses of the psittacosis-lymphogranuloma venereum group(1-3). The large size of the pox viruses, also of the herpes and myxoma viruses, approximating that of the rickettsiae, made it seem worthwhile to evaluate the effect of antibiotics against these agents. Recently, herpes simplex virus has been studied by Baldridge and Blank(4), who demonstrated that its activity *in vivo* and *in vitro* is not inhibited by aureomycin.

The present report deals with our observations on the effect of aureomycin, chloramphenicol and para-aminobenzoic acid, both singly and in combination, on the infections of the chorioallantoic membrane of the embryonated egg with the viruses of infectious myxoma and vaccinia.

Method and materials. Crystalline chloramphenicol (Chloromycetin Lot No. 131334), which was kindly supplied by the Research Laboratory of Parke, Davis and Co., was dissolved just before use in sterile distilled water. In the case of aureomycin the antibiotic for intravenous administration containing 100 mg aureomycin hydrochloride and 50 mg sodium glycinate in a vial was dissolved in sterile physiologic saline. Para-aminobenzoic acid was made up in physiologic saline to a concentration of 2 mg or 4 mg per ml and autoclaved at 10 pounds for 15 minutes. The myxoma virus used was in its forty-first or forty-second chorioallantoic passage. The vaccinia virus was egg-adapted from the dermal strain by 11 serial passages on the chorioallantoic membrane in the laboratory of Dr. G. J. Buddingh of the Louisiana State University Medical School. Virus inoculations were made onto the dropped chorioallantois. In the case of myxoma virus, groups of 9 to 18 11- or 12-day eggs were employed in each experiment and the inoculum varied from 10 to 1000 infectious doses in 0.1 or 0.2 ml of distilled water. In the case of vaccinia virus, groups of six 12-day chick embryos were used in each experiment and the embryos were incubated for two days before the infected chorioallantoic membranes were examined. The inoculum consisted of 1000 infectious units in 0.2 ml physiologic saline. *In vitro* tests were performed by diluting the lyophilized virus-infected tissue to 10^{-2} by weight with solutions containing the desired amount of drug; the mixture was then allowed to stand in the refrigerator for varying periods of time, generally 2 or 4 hours. Serial 10-fold dilutions were prepared and 3 or 4 eggs were inoculated with each dilution. The *in vivo* effect of the drug used was thus largely eliminated. For the *in vivo* tests, the desired doses of drug in solution were injected onto the chorioallantois or into the yolk sac of the embryos. Several different treatment schedules were used to detect the possible prophylactic or therapeutic action of the agents under study. For each experiment, an equal number of control embryos was infected with the same virus preparation used in the test, except that distilled water or physiologic saline was given instead of the drug solution. The system of recording lesions by the gross pathology and number of plaques on the membrane was used(6).

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* This investigation was supported (in part) by a research grant from the National Cancer Institute, of the National Institute of Health, Public Health Service.

† Mead Johnson Fellow in Pediatrics.

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4. Baldridge, G. D., and Blank, H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 560.

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TABLE I. *In vitro* Effect of Chloramphenicol and Para-Aminobenzoic Acid on Myxoma Virus.

Approximate No. of infectious units	Chloramphenicol 2000 γ/ml for 24 hr Treated	 Control
1×10^1	3+*	3+
1×10^2	1+	1+
1×10^3	D	D
	Chloramphenicol 1000 γ and PABA 1000 γ for 4 hr	
1×10^1	3+	4+
1×10^2	1+	1+
1×10^3	D	D

* Mean value for lesions on 3 or more chorioallantoic membranes.

4+: Many discrete lesions and confluent lesions.
3+: >50 discrete lesions and confluent lesions.
2+: >50 discrete lesions.
1+: <50 discrete lesions.
D = Doubtful.

TABLE II. Growth of Myxoma Virus on Chorioallantois of Eggs Treated with Aureomycin, Chloramphenicol and PABA.

Approximate No. of infectious units	Chloramphenicol 1000 γ and PABA 2000 γ/egg Treated	 Control
1×10^1	3+*	3+
1×10^2	3+	3+
1×10^3	D	D
	Aureomycin 1000 γ and chloramphenicol 400 γ/egg	
1×10^1	n.t.†	n.t.
1×10^2	3+	3+
1×10^3	n.t.	n.t.
	Aureomycin 200 γ and PABA 1000 γ/egg	
1×10^1	n.t.	n.t.
1×10^2	4+	3+
1×10^3	n.t.	n.t.

* Mean value for lesions on 3 or more chorioallantoic membranes.

4+: Many discrete lesions and confluent lesions.
3+: >50 discrete lesions and confluent lesions.
2+: >50 discrete lesions.
1+: <50 discrete lesions.
n.t. = not tested.

Results and discussion. A series of 24 therapeutic trials, both *in vivo* and *in vitro*, against myxoma virus infection in the chick embryo was performed. In Tables I and II are summarized the data of a few typical tests. In no experiment could any difference between control and treated membranes be detected, either as to the size or the number of the lesions. In one experiment, not included in the table, in the course of which the membranes were examined every 24 hours after infection, chloramphenicol did not appear to exercise any influence on the course and appearance of the lesions on the membranes.

Our investigations with the vaccinia virus were limited to *in vivo* studies. In agreement with the findings of others(7-9), our preliminary experiments showed that none of the three agents studied, when used singly, was active against the vaccinia virus. Table III presents the results of experiments in which the embryos were infected with vaccinia virus and treated with combinations of the therapeutic agents. In one trial, in which aureomycin and para-aminobenzoic acid were used in combination, the mean value for the lesions on the treated membranes was less than that for the controls. This difference was not statistically significant; moreover, this combination of therapeutic agents failed to delay the death of embryos following infection.

Baldridge and Blank(4) showed that, when the antibiotic was injected onto the chorioallantois, aureomycin is more toxic for the chick embryo than for most other laboratory animals, and that there is a significant dif-

TABLE III. *In vivo* Effect of Aureomycin, Chloramphenicol and Para-Aminobenzoic Acid on Vaccinia Virus.

Combinations of therapeutic agents	Mean No. of lesions on 6 chorioallantois Treated	 Control
Chloramphenicol 500 γ and PABA 1000 γ/egg	9	7.3
Aureomycin 2,000 γ and chloramphenicol 400 γ/egg	17	15.2
Aureomycin 2,000 γ and PABA 800 γ/egg	9.8*	15.2

* According to Student's "t" test, the probability is greater than 0.1.

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TABLE IV. Toxicity of Aureomycin and Chloramphenicol for Embryonated Eggs.

Dose in µg	Route	Aureomycin	Chloramphenicol
1000	Yolk sac	8/24*	2/21
800	Chorioallantois	17/17	
200	”	6/12	2/18

* Numerator: No. of embryos dead within 24 hr after treatment. Denominator: Total No. of eggs injected.

ference in toxicity between different lots. We also found our aureomycin preparation to be toxic for the chick embryo. As can be seen from Table IV, all of the embryos which were treated with 800 µg of aureomycin per egg by the chorioallantoic route died, while most of the embryos tolerated 1,000 µg or 2,000 µg per egg given into the yolk sac. This finding might well be correlated with the observations made by Lépine and his associates(10) that the growth of certain proliferating cells was definitely retarded by 100 µg of aureomycin and chloramphenicol per ml of tissue culture medium, and completely inhibited by 1,000 µg per ml. Moreover, Womack and his associates(11) demonstrated the presence in egg yolk of a substance which inhibits deterioration of aureomycin activity. The number of embryos treated was too small to evaluate the relative toxicity of aureomycin and chloramphenicol for the chick embryo, although aureomycin seemed to be a little more toxic than chloramphenicol.

Conclusion. A study of aureomycin, chloramphenicol and para-aminobenzoic acid in myxoma virus and vaccinia virus infection in the chick embryo failed to show any chemotherapeutic or chemoprophylactic action of these agents, either when they were used alone or in combination. In agreement with others, we also found that aureomycin, when introduced onto the chorioallantois, appeared to be toxic for the chick embryo.

Grateful acknowledgement is made to Miss Barbara Jo Holmes for her help.

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Received September 1, 1950. P.S.E.B.M., 1950, v75.

Influence of Estradiol on P^{32} Uptake by the Uterus. (18294)

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The object of this investigation was to secure increased concentration of a radioactive isotope in a target organ of the female genital tract. Evidence has been adduced that the weight response of target organs in treated animals may be influenced in an unexpected manner by varying the proportions of the constituents of the hormonal mixtures without changing the total equivalent doses(1,2). In order to elucidate the mechanism of such reactions experiments employing radioactive phosphorus (P^{32}) were designed. In the light of the observation that the rate at which new tissue is formed is one of the factors that influences the uptake of phosphorus(3), it seemed reasonable to assume that the uptake of P^{32} by a target organ, such as the

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† Marguerite Singer Milligan Fellow in Cancer Research.

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TABLE I. Effect of Single Injection of Aqueous Estradiol on P^{32} Uptake by Immature Uterus.

Total No. animals	Avg time in hr	Avg uterine wt in g	Stand. error*	% dose × 10^3†	% dose/g‡	Stand. error	Inj.§
4	3	.02	±.005	19	0.92	±.17	P^{32}
10	3	.03	±.003	44	1.26	±.10	” + Est.
4	6	.02	±.002	22	0.95	±.22	”
8	6	.04	±.006	46	1.23	±.09	” + Est.
3	24	.03	±.003	27	1.01	±.26	”
9	24	.05	±.005	77	1.67	±.12	” + Est.
3	48	.02	±.001	15	0.81	±.11	”
8	48	.03	±.003	64	1.86	±.11	” + Est.
2	72	.02		14	0.83		”
9	72	.02	±.002	42	1.75	±.14	” + Est.

* Stand. error $= \sqrt{\frac{\Sigma\Delta^2/n-1}{n}}$.

† % dose $= \frac{\text{c/m sample}}{\text{c/m dose}} \times 100$.

‡ % dose/g $= \frac{\left(\frac{\text{c/m sample}}{\text{c/m dose}} \times 100\right)}{\text{g wt of organ (wet)}}$.

§ Dose employed = 0.3-0.42 γ estradiol
4.0 μc P^{32}.

uterus, might be enhanced if that organ were stimulated by estrogens.

Material and method. Female rats 19 to 23 days old were employed in this study. Our previous investigations(2) indicated that the body weight had no significant effect upon the uterine response to injected estrogens. All injections, both of P^{32} and of estrogens, were made subcutaneously. The first experiment (Table I) comprised a group of 60 animals which were divided into 2 groups; those which received estradiol plus P^{32} and, those which received P^{32} alone (injected controls). The estradiol was administered in 20% alcoholic solution in doses ranging from 0.3 to 0.42 γ of estradiol. It was found(2) that this dose of estradiol gave the maximum uterine weight increment response. These animals received simultaneously 4.0 μc of P^{32}. The injected control animals received 0.5 cc of a 20% alcoholic solution containing P^{32} only. The animals were killed with chloroform at 3, 6, 24, 48 and 72 hours after injection. The uteri were immediately dissected, excised and spread on tared radiation planchettes. They were promptly weighed on a chainomatic analytical balance with magnetic damping and then dried under an infra-red lamp. In most cases the uteri were counted using a thin (2 mg/cm²) mica window counter, but a few were counted with a glass tube. All counts were corrected for background and radioactive decay. Counts agreed before and after rotation of the planchettes through 90°.

A second group of animals (Table II) was employed in order to determine whether the radioactive phosphorus became bound in the cell and did not represent a water transport. Thirty-six animals were treated in the same manner as the aforementioned group. Two groups were employed and were designated as "run 1" and "run 2." For purposes of fractionating, the uteri were removed in pairs and placed in a tared homogenizer (double tube type). They were weighed and placed in a dry ice acetone bath. The uteri were then ground with emery and 1 ml of water. 1.0 to 1.2 ml of 10% trichloracetic acid (TCA) was added and the mixture was filtered through No. 50 Whatman paper in a stainless steel holder, and washed twice with 2 cc portions of 2% TCA containing NaH_2PO_4 (3 mg P/ml). The precipitates and paper were digested with 15 ml concentrated HNO_3 to small volume, then treated with 2 ml of concentrated HCl and water. After making up to volume the TCA filtrates and insoluble fractions were counted using a dipping counter.

A third group of 16 animals was employed in order to determine the effect of increasing the weight of the uterus on the uptake of P^{32}. The increased uterine weight in this group was accomplished by injecting the estradiol in oil in divided doses (Table III). These animals were given a total of 0.5 γ of estradiol in oil, divided into 3 daily injections. On the first

TABLE II. Fractionation of Uteri into Acid-Soluble and Acid-Insoluble Portions.

Total No. animals	Run No.	Avg time in hr	Avg uterine wt in g	Acid-soluble % dose × 10^3	Acid-soluble % dose/g	Acid-insoluble % dose × 10^3	Acid-insoluble % dose/g	Inj.
2	1	3	.04	18	.47	10	.26	P^{32}
2	1	3	.06	35	.59	17	.28	,, + Est.
2	1	6	.03	18*	.58*	6	.20	,,
2	2	6	.04	17	.43	8	.21	,,
2	1	6	.06	9	.16	23	.38	,, + Est.
2	2	6	.06	51	.86	16	.27*	,, + Est.
2	1	24	.04	6	.17	22	.59	,,
2	2	24	.04	17	.46	14	.36	,,
2	1	24	.06	21	.32	56	.86	,, + Est.
2	2	24	.08	38	.45	79	.93	,, + Est.
2	1	48	.04	9	.21	13	.33	,,
2	2	48	.03	9	.26	10	.30	,,
2	1	48	.07	21	.31	60	.89	,, + Est.
2	2	48	.05	22	.43	44	.87	,, + Est.
2	1	72	.03	8	.24	12	.35	,,
2	2	72	.04	8	.18	15	.33	,,
2	1	72	.05	9	.32	43	.91	,, + Est.
2	2	72	.04	15	.38	28	.69	,, + Est.

* Some loss.

TABLE III. Effect of P^{32} Uptake by Increasing Uterine Weight Through Priming with Estradiol in Oil.*

Total No. animals	Avg uterine wt in g	Standard error	% dose × 10^3	% dose/g	Stand. error	Injection†
6	.029	±.002	23	0.79	±.07	P^{32}
10	.105	±.003	139	1.33	±.06	P^{32} + Est.

* Animals killed 72 hr after inj. with P^{32}.
† Total dose employed = P^{32} 5 μc.
Estradiol 0.5 γ.

day of the injection of the estradiol, 5 μc of P^{32} was injected separately in an aqueous solution. They were then injected for 2 more days with estradiol in oil and were killed on the fourth day with chloroform.

The livers were removed in one group of animals (Table IV) with a view toward determining the uptake of P^{32} by the liver in estradiol treated animals. The same methods were employed in this group as in the previously mentioned groups of rats.

Discussion. The estrogenic hormones are known to have a stimulating effect on vaginal mucosa, endometrial epithelium as well as on the acinal epithelium of the breast. The proliferation thus produced would tend to increase the uptake of phosphorus made available to the animal organism. This idea received some support from the preliminary report of Hertz(4) who observed an augmented uptake of P^{32} in cancer tissue of the breast in humans who had been treated previously with testosterone because of breast malignancy.

The data in Table I indicate that early in the experiment the average uptake of P^{32} was greater in the uteri of the rats treated with estradiol plus P^{32} than in the rats that received P^{32} alone. The uptake of P^{32} by the estradiol plus P^{32} treated rats increased with time, reaching the highest concentration at the end of 48 hours. On the other hand, the uteri of the animals that were injected with P^{32} alone showed significantly lower activity. The concentration of P^{32} in these uteri remained fairly constant for the duration of the experimental time.

The data recorded in Table II indicate that there is increased binding of the administered P^{32} within the target organs of animals treated with estradiol. The acid insoluble fraction in the estradiol treated rats shows low activity in the first few hours. There is a rapid increase in the first 24 to 48 hours that is well maintained through 72 hours. When P^{32} is injected alone the per cent dose per gram of

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TABLE IV. Uptake of P^{32} by the Liver in Estradiol Treated Animals.

Time in hr	% dose/g	Injection
6	2.23	P^{32}
6	1.85	" + Est.
24	1.57	"
24	0.98	" + Est.
48	1.19	"
48	1.76	" + Est.
48	2.47	"
48	2.28	" + Est.

tissue in the acid insoluble fraction is lower in the early hours than when P^{32} and estradiol are injected together. The acid soluble fraction, when P^{32} is injected alone, shows higher activity in the first few hours than does the acid insoluble fraction. It then drops to a fairly constant level which is maintained through 72 hours. No evident explanation for this is apparent.

In order to determine whether we could increase the uptake of P^{32} by further increasing the weight of the uterus an experiment was designed toward that end. Estradiol in oil was injected for three successive days, in place of a single injection of aqueous estrogen, so that an average uterine weight of 104 mg was accomplished (Table III). In spite of a 4- to 5-fold weight increment in the uterus of the animals thus treated the uptake of P^{32} by the organ was no greater than in those animals (Tables I and II) with smaller uteri in consequence of a single injection of aqueous estradiol.

Since Zondek(5) has shown that estradiol is inactivated in the liver the question arose whether the combined use of P^{32} and estradiol may be reflected in that organ. Reference to Table IV indicates that there is no preferential localization of P^{32} in the liver. The process of the intermediary metabolism of estradiol apparently plays no part in the localization of the P^{32}. When, however, the estradiol is instrumental in stimulating cells to proliferation, as in a target organ such as the uterus and the breast, the phosphorus becomes incorporated within the cells in greater concentrations than under normal conditions.

The data in this report suggest the important possibility that therapeutic agents having a short half life might be expected to exercise an inhibitory effect on certain neoplastic tissue if the latter is a part of a target organ, the growth of which can be stimulated by a specific agent. P^{32} emits the beta ray which has the capacity to produce ionization in tissues and has a maximum range of penetration of approximately 7 mm(3). We are currently employing the combined use of a specific steroid with P^{32} as a means of therapy in malignancy of the genital tract. The evaluation of the application of this principle awaits further study.

Summary. 1. The preferential localization of radioactive phosphorus is enhanced in the uterus of the immature rat by the administration of estradiol. 2. P^{32} becomes bound in the estradiol primed uterus as evidenced by the increase of P^{32} in the acid insoluble fraction. 3. The liver is not influenced in this process, indicating that the proliferating cells in a hormone treated target organ influence the uptake of P^{32}. 4. The data indicate a suggested approach to the treatment of genital malignancies.

The authors wish to thank Dr. Campbell Moses, Jr., of the Addison Gibson Laboratory of Applied Physiology of the University of Pittsburgh for the P^{32} used in this investigation.

Received September 11, 1950. P.S.E.B.M., 1950, v75.

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Similarity of Hematologic Effect of Pyridoxine Deficiency, Cortisone, and Myeloid Metaplasia Factor of Human Urine.* (18295)

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Previous work done in this laboratory and studies reported by others have shown that similar hematologic effects have been produced in human beings or animals by pyridoxine deficiency induced by desoxypyridoxine, by cortisone and adrenocorticotropic hormone, and by a myeloid metaplasia factor recoverable from human urine in certain disease states. More detailed experiments have been performed in mice to study further the similarity of effect produced by these apparently unrelated compounds and to discover whether there is any evidence that the effects are mediated through a common mechanism.

Pyridoxine deficiency. A previous study (1) in mice showed that pyridoxine deficiency was associated with granulocytosis and lymphopenia. The regimen used produced only a partial deficiency, however, as evidenced by the absence of weight loss, anemia, lymphoid atrophy and convulsions. A more complete deficiency was produced in the present experiment by administering the desoxypyridoxine† orally rather than parenterally. It was added to the Purina dog chow diet in percentages of 0.05%, 0.1% and 0.15% and given *ad libitum* to 3 groups of 5 young adult Swiss mice. These animals ate 3 to 6 g daily so that the 3 groups received 1.5-3 mg, 3-6 mg, and 4.5-9 mg daily of desoxypyridoxine, respectively. Hematologic changes occurred in all 3 groups within 5 days. Granulocytosis and lymphopenia were equally great in all groups indicating that the smallest amount of desoxypyridoxine administered produced maximal results. Three mice died on the 18th, 23rd and 31st days. By the 32nd day the other 12 had significant weight loss. Six of these which had moderately severe dermatitis were killed and autopsied. The remaining 6 animals were continued on the diet containing 0.05% desoxypyridoxine to which was added 0.15% of pyridoxine HCl, providing each animal with 4.5 to 6 mg of pyridoxine daily. The blood picture was restored to normal promptly and weight gain occurred. Definite tissue abnormalities were present in the animals having dermatitis, in contrast to negative findings in the previous experiment(1) (Fig. 1b). There was slight enlargement of the spleens with extensive infiltration of the pulp by neutrophils and large, immature, mononuclear cells, with occasional mitoses. Megakaryocytes were slightly increased in number and erythropoietic elements were abundant. Atrophy of the splenic follicles did not occur. Changes in the liver were not striking, only a few small areas of infiltration with neutrophils and mononuclear cells being present.

Atrophy of lymphoid tissue might have been expected(2), but none occurred. In addition to the intact splenic follicles, the thymus and lymph nodes were of normal size and histologic structure. Mild adrenal cortical hyperplasia was evidenced by a measurable increase in the width of the cortex as compared with normal animals.

Cortisone effect. The occurrence of lymphopenia, granulocytosis and regression of lymphoid tissue associated with administration of ACTH and cortisone has been described(3-8). To compare this effect with

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* These studies were aided by a grant from the National Vitamin Foundation.

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† Kindly furnished by Merck and Co.

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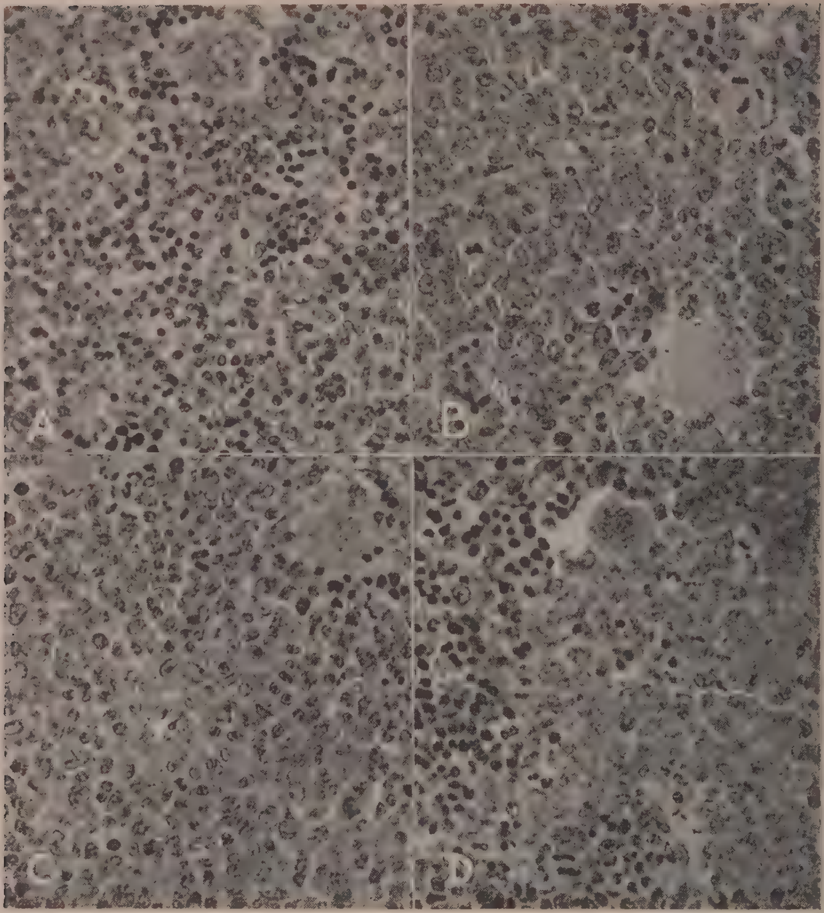

Fig. 1. Spleens. ×370.
(A) Normal. (B) Pyridoxine deficiency. (C) Cortisone. (D) Urine factor.

that produced by pyridoxine deficiency, 5 normal mice were given 1 mg of cortisone acetate daily in a single subcutaneous injection. One died on the 4th day. Within 5 days the mean lymphocyte count of the remaining 4 decreased from 15,250 to 4,750 per cmm and remained below this level during the remaining 25 days of the experiment. During the same time, the mean granulocyte count rose from 4,250 to 10,500 per cmm, the highest recorded count being 38,400 per cmm. Unlike the lymphocyte counts the granulocyte counts showed considerable day to day variation. Three animals lost 1.9, 2.2, and 0.9 g in weight, respectively; one gained 0.1 g. After 30 days of cortisone administration the animals were killed and autopsied. The spleens were uniformly about half normal size and there was extensive cellular infiltration of the pulp identical with that occurring in the pyridoxine deficient mice (Fig. 1c). In 2 mice, the splenic follicles were smaller and less ac-

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tive than normal while in the other 2 the follicles were normal. There were no significant changes in the livers or marrows. Marked narrowing of the adrenal cortex with reduction in size and granularity of the cells was evident, with advanced atrophy of the cortex in one animal.

Myeloid metaplasia factor from human urine. The production of myeloid metaplasia in guinea pigs by the injection of fractions of urine from patients with chronic myeloid leukemia has been described(9). Subsequent reports(10-14) confirmed this finding and showed, in addition, that the active material was also present in the urine of certain individuals not having chronic myeloid leukemia. Preliminary experiments(14) indicate that mice developed similar but more pronounced lesions than guinea pigs. Urine fractions known to have produced changes in guinea pigs have now been administered to 263 mice. The test solutions were prepared from urine by a benzoic acid adsorption technic(15) and further fractionated as previously described(13). The mice received a total of 5 ml of solution derived from 500 ml of urine in 10 daily subcutaneous doses of 0.5 ml. Leukocyte and differential counts were done at the beginning and end of the 10-day period. The animals were killed and autopsied on the 10th day.

Polymorphonuclear leukocytosis of greater degree than that previously produced in guinea pigs, occurred regularly in the mice. The degree of leukocytosis correlated well with histologic changes in the hematopoietic organs. In one group of 39 mice the mean granulocyte count rose from 3,200 to 30,000 per cmm. Unlike the pyridoxine deficient and cortisone treated animals, there was no change in the mean lymphocyte count, the mean control count being 10,600 per cmm as compared with 10,800 per cmm at the conclusion of the experiment.

Changes occurred in the spleen, liver and bone marrow. The spleen size increased 3 to 4 times. The number and size of the follicles and the activity of the germinal centers was diminished. The pulp (Fig. 1d) was infiltrated with neutrophils, and large, immature mononuclears showing mitoses. The latter cell often predominated. Megakaryocytes and nucleated red cells were greatly increased in number. The changes were qualitatively identical with those produced by cortisone and pyridoxine deficiency, differing only in the degree of infiltration and regression of the follicles.

In the liver, 2 types of infiltration occurred, the first characterized by portal and perivascular infiltration by neutrophils and medium-sized and large mononuclear cells having occasional mitotic figures. In the second type, the same cells were irregularly distributed throughout the parenchyma, in a morphologic pattern identical with that occurring in human chronic myeloid leukemia. There was an increase in the neutrophil and immature mononuclear cells in the marrow, with a decrease in nucleated red cells. Cortisone and pyridoxine deficiency produced only minimal changes in the liver and none in the marrow.

Mild hyperplasia of the adrenal cortex, similar to that induced by pyridoxine deficiency, occurred. Regression of lymphoid tissue did not occur. The thymus and lymph nodes were normal in size and histology.

While urines from normal individuals consistently contain little or none of the myeloid metaplasia factor, it is impossible to correlate the amount present in pathologic urines with the disease state of the patient. The substance cannot be isolated from the urine of all patients with chronic myeloid leukemia and it may be present in large amounts in the urine of patients with such

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conditions as carcinoma, fever, chronic lymphoid leukemia and monocytic leukemia. These comments refer to results obtained with the isolation procedure described above, which are not necessarily comparable to the results obtained with the fat soluble fractions of Miller and Hause(12).

Discussion. Similar hematologic and histologic changes occur in mice as the result of pyridoxine deficiency, cortisone administration, and administration of a myeloid metaplasia factor from human urine. Granulocytosis and myeloid metaplasia were constant features of the 3 conditions. The splenic infiltration was not reported in other experiments(16,17) in which mice received 1.25-2.5 mg of cortisone daily for periods of 6-9 days. Lymphopenia occurred with cortisone administration and pyridoxine deficiency, but was not produced by the urine fractions. Unlike animals treated with adrenal cortical extract(4) or humans given ACTH(8), the lymphopenia which occurred in 2 of the 3 groups persisted for the 30-32 days of these experiments instead of disappearing during the course of the experiment. In adrenalectomized animals(4) and in humans with adrenal insufficiency(8), ACTH produced granulocytosis without lymphopenia, a change similar to that produced by the urine fractions.

In our experiments with cortisone the lymph nodes and thymus were normal in size and histology, without regression of lymphoid tissue. It has been suggested(18) that prolonged administration of cortisone might produce an initial regression followed by compensatory regeneration of lymphoid structures. If this occurred, it was not reflected in the peripheral blood, since the lymphopenia, once established, persisted unchanged.

The fact that similar changes occurred with 3, apparently unrelated substances, suggests the possibility that some common mechanism is operating in the 3 conditions. In previous work(14) with the urine fractions it was considered that the active material might be a regulatory hormone, in which event a high degree of structural specificity and some correlation between the disease state of the patient and the amount of material in the urine might be expected. Alternatively, the factor might be one of a group of substances which, although differing widely in chemical structure, could alter the production of a regulatory hormone, which in turn would produce the hematopoietic changes. The fact that similar changes occurred in all 3 groups in the present experiment lends support to this hypothesis. It should be noted that significant differences did occur, however. The urine fractions did not produce lymphopenia. With pyridoxine deficiency the hematologic changes were maintained for 30 days, a result not characteristic of the alarm reaction, in which there is a reversal toward normal within a brief period of time. The fact that the effects of pyridoxine deficiency could be completely abolished by the addition of pyridoxine to the diet while continuing desoxypyridoxine indicates that the effect of this chemical is exclusively that of a displacer of the active B_6 vitamin and that, in itself, it does not activate the alarm reaction.

While it is possible that the effects of pyridoxine deficiency might be mediated by the alarm reaction, it seems just as valid to consider the possibility that the hematopoietic results of cortisone administration might be the result of the establishment of an effective deficiency of the active vitamin B_6 coenzyme, occurring at some stage in the metabolic cycle with which the vitamin is concerned.

Summary. Similar hematologic and histologic changes occurred in mice made pyridoxine deficient, in mice treated with cortisone, and in mice treated with a myeloid metaplasia factor from human urine. It is concluded that the changes produced by pyridoxine deficiency and by the urine fractions are not necessarily mediated through the mechanism of the alarm reaction. Other mechanisms of action are considered.

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Received September 19, 1950. P.S.E.B.M., 1950, v75.

Cerebrosides from Spleen and Brain from an Adult with Gaucher's Disease. (18296)

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The author(1) demonstrated that the cerebroside from the spleen obtained from an adult female with Gaucher's disease contained glucose rather than galactose. Similar reports have been reported by other workers (2-5). It appears that while the glucasido-form of cerebroside may not always be found in the spleen from a case of Gaucher's disease, such formation is peculiar to the Gaucher cells.

The present report deals with results of analyses of brain and spleen tissues obtained, within a few hours after death, from a 58-year-old female. The case presented certain anomalies but autopsy findings confirmed that the patient undoubtedly had Gaucher's disease.*

Analytical methods and results. Isolation of the cerebrosides. The cerebrosides were isolated by the methods described in the earlier work(1). The yields were not large but the materials appear to be pure, as shown by solubilities, melting points and nitrogen concentration. Total nitrogen was determined by the micro method of Tompkins and Kirk(6).

Identification of the carbohydrates. It was found in the earlier work(1) that if carbohydrate alone was to be split off from the cerebroside, the Kirk(7) semi-micro method yielded nearly theoretical results and good recoveries of added glucose and galactose. Therefore, this method was employed in the present work. Determinations were made of "free sugar" (reduction before hydrolysis), total carbohydrate found after hydrolysis and residual sugar following hydrolysis and yeast fermentation. The Somogyi modified blood sugar method(8) was employed. Determinations were by spectrophotometer† and results calculated from curves obtained with standard solutions of glucose and galactose.

Mucic acid tests. Solutions of the cerebrosides were hydrolyzed and tests for mucic acid carried out on the filtrates.

Results of the analyses are presented in Table I. The losses of carbohydrate following fermentation demonstrated the presence of the glucasido-form of cerebroside from the spleen and the normal galactosido-cerebroside from the brain.

TABLE I. Cerebrosides from Spleen and Brain from an Adult with Gaucher's Disease.

	Spleen	Brain
Cerebrosides		
MP (178°C)	175, 176°C	176, 178°C
Nitrogen (1.73%)*	1.82, 1.92%	1.60, 1.68%
Free sugar %	1.2, 1.06%	0.8, 1.2%
Following hydrolysis		
Mucic acid test	Neg.	Pos.
Carbohydrate—% (22.2)*	15.1, 16.6%	19.0, 18.1%
Loss on fermentation	95.4, 96.0%	2.5, 5.5%

* Theoretical value.

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† Coleman Jr., Model No. G.

Received September 25, 1950. P.S.E.B.M., 1950, v75.

Production of Hypertension Following Choline Deficiency in Weanling Rats.[*] (18297)

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Recently, Hartroft and Best(1,2) have demonstrated that weanling rats surviving the renal damage resulting from a brief period of choline deficiency(3) have a high incidence of hypertension. Sobin and Landis(4) did not find any evidence of hypertension in weanling rats during acute choline deficiency or in rats maintained on a choline-deficient diet over long periods of time. This study was undertaken to determine the effect upon blood pressure at maturity of varying degrees of choline deficiency for a 5-day period after weaning and to observe the effect of a similar choline deficiency on more mature rats.

Methods. Three groups of weanling rats of the Sprague-Dawley strain (average weight 43 g) were fed (1) a choline-free diet,[†] (2) the same diet plus 400 mg choline chloride per kg diet, and (3) with 2 g of choline chloride per kg added to the diet for a period of 5 days. At the end of this time representative animals from each group were sacrificed and the remaining animals restored to a normal diet (Rockland Rat Ration) for 6 months until sacrifice. During this period indirect blood pressure determinations were made by the method of Kersten, *et al.*(5). Under nembutal anesthesia at the time of sacrifice direct blood pressure readings were obtained by cannulation of the abdominal aorta. Three groups of older rats of the same strain (average weight 140 g) were given similar diets for 10 days followed by sacrifice of representative animals and the administration of a normal diet for 6 months.

Results. The data obtained are summarized in Tables I and II. It will be noted that approximately 70% of the weanling rats surviving the 5-day period of a choline-free diet had arterial blood pressure levels over 150 mm Hg six months later. In contrast to this hypertension did not develop in the animals fed 0.4 mg or 2.0 mg of choline for the brief experimental period. Similarly, hypertension did not occur in the groups of older rats fed either a choline-free or low-choline diet for 10 days. It should be noted that the weanling rats on a choline-free diet had an average weight gain of 7.4 g during the 5-day experimental period, whereas the older rats failed to gain in weight during the experimental period.

Summary. Of 28 weanling rats surviving a 5-day choline-deficient diet and maintained on a normal diet for 6 months thereafter 20 animals or 71% had arterial blood pressure levels over 150 mg at the time of sacrifice. Similar animals receiving an 0.4 mg % or 2.0 mg % choline diet and older rats (140 g) surviving 10 days of similar diets did not have demonstrable hypertension at sacrifice 6 months later.

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[*] This study was made possible by a grant from the American Foundation for the Study of High Blood Pressure. Support was also received from the Sarah Mellon Scaife Foundation.

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[†] Basic diet per kg

	g
Sucrose	455
Lard	300
Casein (vitamin test)	100
Mazola	50
H-E Cellulose	50
Salt mixture	40
l-Cystine	5
Natola	200
Plus daily vitamin supplement tablet	
	mg
Thiamine chloride	.04
Calcium pantothenate	.2
Pyridoxine	.05
Niacin	.1

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TABLE I. Blood Pressure Findings in Rats Surviving % Day Period of Choline Deficiency Shortly After Weaning.

Diet	No. of animals	Avg initial wt, g	Avg wt after 5 days on low choline diet, g	Avg wt at 6 mo., g	Avg blood pressure at sacrifice, mm Hg	Animals with blood pressure over 150 mm Hg %
Choline free	28	43.4	50.8	395.4	166 (100-256)	71
0.4 mg % choline	20	43.7	54.9	411.4	118 (95-160)	5
2.0 mg % choline	22	42.9	54.7	392.8	114 (70-145)	0

TABLE II. Blood Pressure Findings in Older Rats Surviving 10 Day Period of Choline Deficiency.

Diet	No. of animals	Avg wt at start of low choline diet, g	Avg wt after 10 days on low choline, g	Avg wt 6 mo. later, g	Avg blood pressure at sacrifice	Avg heart: body wt %	Avg liver: body wt %	Avg kidney: body wt %
Choline free	24	140.2	140.6	353.2	100 (70-128)	.337	3.182	.748
0.4 mg % choline	20	135.1	134.8	346.5	120 (104-144)	.330	3.345	.740
2.0 mg % choline	22	145.6	141.2	324.9	110 (96-136)	.365	3.070	.763

Received October 5, 1950. P.S.E.B.M., 1950, v75.

Leukocytic and Pyrogenic Effects of Typhoid Vaccine and Augmentation by Homologous Plasma. (18298)

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The response of a rabbit to the intravenous injection of a moderate amount of pyrogenic solution is characterized by an initial leukopenia followed by a leukocytosis and hyperthermia. There are varying opinions as to which of these reactions is the best indicator for the presence of pyrogens. Chapman(1) and Bandelin(2) have reported a leukopenia lasting 45-90 minutes following the intravenous injection of pyrogens into rabbits as a reliable test of pyrogenicity. Young and Rice(3) have reported that in dogs, a leukocytosis during the third to sixth hours after injection is a more reliable index of pyrogenicity. Co Tui and Schrift(4) have proposed a pyrogen test using both rabbits and dogs. If there is a positive thermal response in the rabbit, the test is repeated in dogs where a leukopenia (fall of 5000 cells) and a rise in temperature of 0.5°C in 1-3 hours is considered a positive test for pyrogen. A negative thermal response in rabbits is considered of more significance than a positive response. The same authors(5) feel that the phenomenon of leukopenia which accompanies the pyrogenic reaction seems to be more sensitive in dogs and affords an added check. Bose and Ahuja(6), among others, did not find the leukocyte changes in rabbits as reliable as the temperature changes. A comprehensive review of the leukocyte response to pyrogenic agents and nonspecific protein injections prior to 1922 is included in the text by Petersen(7).

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The objective of this work was to make a comparison of the leukocytic and thermogenic effects of pyrogens on rabbits with particular emphasis upon the correlation between the amount of pyrogen administered and the magnitude of the response. Early in the course of this study it became apparent that the addition of plasma to the pyrogen altered the effects observed and, therefore, a comparison of the responses obtained with plasma and saline as menstrua was also made.

Materials and methods. Two strains of *Eberthella typhosa* were used as the source of pyrogen in these experiments. The Naval Medical Research Institute strain 901 W *E. typhosa* was employed in most of the work. A lyophilized culture was grown on brain heart-infusion agar slants which were incubated at 37°C for 24 hours. The growth of each slant was washed off with sterile normal saline and the organisms were killed by placing the tubes containing the suspensions in boiling water for 15 minutes. The sterile individual suspensions were pooled and a bacterial count performed using the Petroff-Hausser bacterial counting chamber. A sterile pyrogen-free saline solution (0.85% sodium chloride) was then added to the suspension to give the desired dilution of 125 million organisms per ml. No chemical preservative was added. A few additional studies were done using strain 58 *E. typhosa* obtained from the Army Medical Center, Washington, D. C. It was cultured on tryptose phosphate agar, and was killed by immersing a saline suspension in water at 58°C for 60 minutes.

The plasma was obtained by pooling blood from 5 previously untreated rabbits. Sodium citrate (0.4%) was used as the anticoagulant. Insofar as possible, the plasma was prepared under conditions that simulate the technic used for human plasma production(8). Prior to use, each lot of pooled plasma was cultured and found sterile. The saline injected was isotonic, sterile and non-pyrogenic. A standardized method of preparing the plasma-typhoid and the saline-typhoid suspensions was employed throughout the study. One ml of various dilutions of the stock vaccine was added to a tube containing 9 ml of plasma or saline. For the plasma controls, 1 ml of sterile saline was added to 9 ml of plasma. The tubes were agitated to assure thorough mixing and then stored at 5°C for 12-18 hours. In addition to the precaution of culturing the plasma and vaccine prior to mixing, some experiments were done in which the remaining portion of each solution injected was cultured and found to be sterile. Prior to injection, all solutions were warmed to 37°C and the volume given was always 5 ml.

All glassware and needles used in these experiments were sterile and pyrogen free. This was accomplished by placing the glassware in a chromic acid bath and the needles in a protein digesting solution for at least 24 hours. After a triple rinse in distilled water, each piece of equipment was wrapped in muslin and exposed to 170°C dry heat for 2 hours.

Fifty-eight New Zealand white rabbits were used in this study. The rabbits were males, approximately 6 months of age, and weighed between 3.0 and 3.5 kg. Each rab-

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bit was used on only one occasion. The animals were studied under conditions controlled with regard to the amount of handling, the environmental temperature and humidity. A standardized technic for taking the leukocyte counts and temperatures was employed. A previous paper gives the details of this procedure and, in addition, describes the leukocyte and temperature variations observed in untreated animals while being studied under these conditions(9). Four basic groups of animals were studied. One group received saline alone, one received saline plus typhoid vaccine, one received plasma alone, and one received plasma plus typhoid vaccine. The volume injected was constant, but the amount of typhoid vaccine was varied as described later. The animals were studied for 6 consecutive hours. Total and differential leukocyte counts were performed every 30 minutes for the first 4 hours and every 60 minutes for the last two hours. In the differential counts, both the monocytes and the lymphocytes were classified as mononuclear leukocytes. Rectal temperatures were taken each hour. The injections were given at the end of the first hour and the leukocyte counts and temperatures taken prior to that time were used as control values. Mean hourly values for the leukocyte counts and temperatures were calculated by pooling the data obtained from each rabbit of a group receiving the same treatment.

Results. Results from representative groups are presented graphically in Fig. 1-5.

Fig. 1 demonstrates the variations found in the leukocyte population and in the temperature of two groups of control rabbits: one group injected intravenously with 5 ml homologous plasma and the other group with 5 ml isotonic saline. There was no essential difference between the response of the two groups. The changes seen in both the plasma and saline injected animals are within the range of variation known to occur in untreated rabbits under otherwise similar conditions(9).

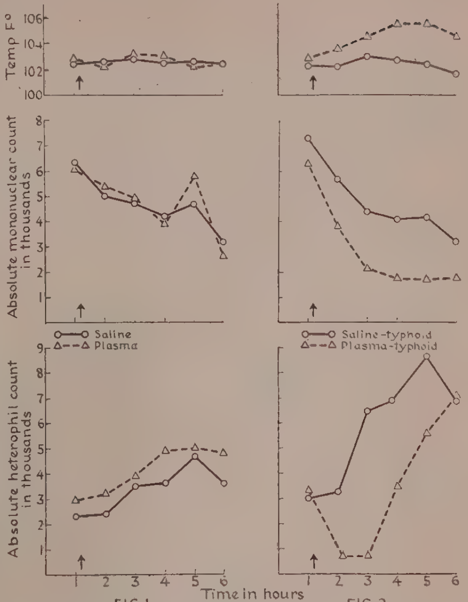

FIG.1 FIG.2

Fig. 1. Mean temperature and leukocyte response of two groups of 4 rabbits injected intravenously with 5 ml 0.85% saline and with 5 ml homologous plasma.

Fig. 2. Mean temperature and leukocyte response of two groups of 4 rabbits injected intravenously with 5 ml 0.85% saline containing 6.25 million *E. typhosa* organisms (strain 901W) and with 5 ml homologous plasma containing 6.25 million *E. typhosa* organisms (strain 901W).

Fig. 2 represents the effect of 6.25 million *E. typhosa* (901 W) organisms added to homologous plasma and isotonic saline. When plasma was used as the menstruum, a different leukocyte and temperature response resulted as compared to the saline suspensions. The animals given the plasma-typhoid solutions developed high fever, an initial heterophile leukopenia, and a secondary heterophile leukocytosis. The rabbits given the saline-typhoid had only an initial heterophile leukocytosis and very little temperature elevation. Both groups developed a mononuclear leukopenia, but the animals given the plasma typhoid injections demonstrated a proportionately greater fall in the mononuclear count than did the animals given the saline-typhoid. The mononuclear leukocyte and temperature changes of these two groups of rabbits suggest that the plasma augments the effects

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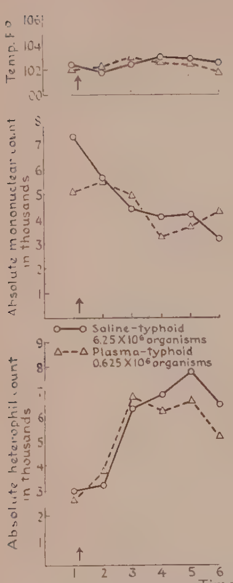

FIG.3

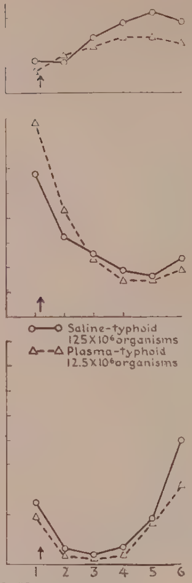

FIG.4

FIG. 3. Mean temperature and leukocyte response of two groups of 4 rabbits injected intravenously with 5 ml 0.85% saline containing 6.25 million *E. typhosa* organisms (strain 901W) and with 5 ml homologous plasma containing 0.625 million *E. typhosa* organisms (strain 901W).

FIG. 4. Mean temperature and leukocyte response of two groups of 4 rabbits injected intravenously with 5 ml 0.85% saline containing 12.5 million *E. typhosa* organisms (strain 901W) and with 5 ml homologous plasma containing 12.5 million *E. typhosa* organisms (strain 901W).

seen in saline and that the effect is, therefore, merely quantitative. However, the heterophile response in this experiment could be interpreted as meaning that the two types of leukocyte changes seen in the plasma and saline animals are of a different character and that the reactions vary qualitatively.

A number of groups of animals were used to test the hypothesis that if the difference between the temperature and leukocyte response in the two groups were only quantitative, it should be possible to produce both types of response with either saline or plasma by altering the amount of typhoid vaccine given. Fig. 3 demonstrates that an initial heterophile leukocytosis can be produced with plasma-typhoid suspensions by reducing the number of organisms injected, and Fig. 4 shows that the initial leukopenia can be produced with saline suspensions by increasing the number of organisms. This demonstrated that differences in the leukocyte response are quantitative. Approximately ten times the number of organisms were required in the saline suspension to produce effects comparable to those obtained with a given number of the same organism suspended in plasma. This 1:10 ratio was noted in all groups studied except when high doses were employed. When the amount of vaccine injected became greater, the difference between the plasma and saline groups disappeared since the leukocyte and temperature changes of both groups reached what appeared to be maximum.

Fig. 3 and 4 also show that the fever response is similarly augmented. In addition, the animals given the bacteria suspended in plasma developed a fever approximately one hour earlier than the rabbits given the saline-typhoid suspensions.

A few groups of animals were studied with a new vaccine made from strain 58 *E. typhosa.* Fig. 5 demonstrates that the temperature and leukocyte effect of the plasma-typhoid and saline-typhoid suspensions made with strain 58 were essentially the same as were found

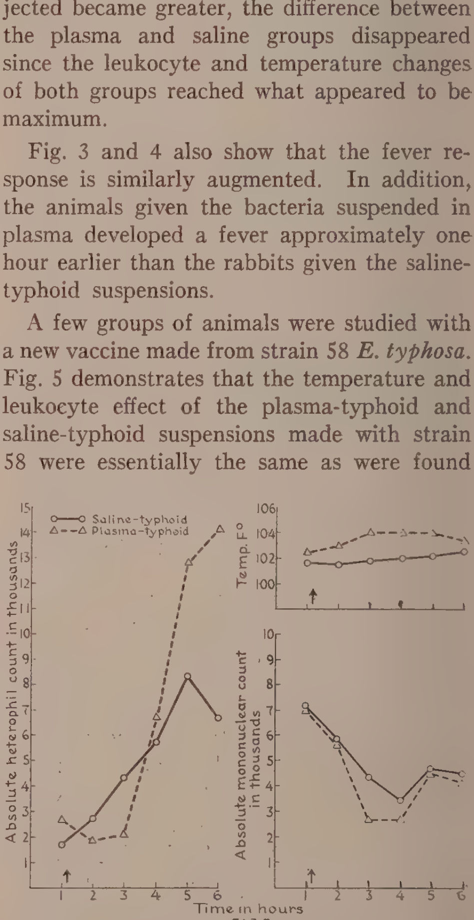

FIG.5

FIG. 5. Mean temperature and leukocyte response of two groups of 4 rabbits injected intravenously with 5 ml 0.85% saline containing 3.1 million *E. typhosa* organisms (strain 58) and with 5 ml homologous plasma containing 3.1 million *E. typhosa* organisms (strain 58).

when using strain 901W, although strain 58 appeared to be more active in producing the leukocyte and particularly the temperature changes. In spite of the relatively potent thermogenic properties of strain 58, the leukocyte changes were still the most sensitive indicator to detect the presence of the vaccine in either saline or plasma. This is demonstrated in the saline-typhoid data in Fig. 5. The leukocyte response was obvious, but the fever response was questionable.

Discussion. The present data and previous literature indicate that there are at least 5 variables which must be considered when using the leukocyte count for a pyrogen test:

1. *Amount of pyrogen given.* Our data with *E. typhosa* agree with the reports(6,7) that the temperature and leukocyte response in the rabbit following the intravenous administration of various numbers of organisms is dependent upon dosage. A relatively small number of typhoid organisms cause very little fever, a heterophile leukocytosis, and a mononuclear leukopenia. When larger doses of vaccine are given, the rabbits respond with a high fever, an initial heterophile leukopenia followed by a secondary leukocytosis, and a mononuclear leukopenia. It therefore follows that a leukopenia alone is an unreliable criterion for pyrogenicity, since small amounts of pyrogen can produce an immediate leukocytosis and a leukopenia is not a part of the reaction.

2. *Time at which the observations are made.* The plasma-typhoid data of Fig. 5 demonstrate the need for frequent serial sampling of the leukocyte population to detect pyrogenic solutions, especially if total leukocyte counts are used as the criterion. If only one count had been made after the injection, it could appear that the total count had increased, decreased, or remained unchanged, depending upon the time interval at which the count had been taken. Since the leukocyte count can change so rapidly from the initial leukopenia to the secondary leukocytosis, and since the time at which the rapid increase occurs is dependent upon the amount of pyrogen given, "spot" determinations at a given time interval after the injection are not reliable.

3. *The differential composition of the leukocyte population.* Situations arise when even serial sampling of the total leukocyte count is not reliable. Such a situation, which is not uncommon, is demonstrated in the saline-typhoid data of Fig. 2. By adding the absolute heterophile and mononuclear values of the group receiving saline-typhoid, it can be seen that the total count was approximately 10, 9, 10, 11, 13, and 10 thousand per cu mm during the 6 hours of study. The total counts similarly calculated from the saline control group in Fig. 1 are 8, 7, 8, 7, 9, and 7 thousand per cu mm. The increase above the initial total count observed in this particular saline-typhoid group is not sufficiently greater than the increase observed in the control animals to justify an interpretation that the test was definitely positive. However, consideration of the absolute heterophile and mononuclear count clearly indicates that a pyrogenic substance was present.

4. *Variety of bacteria used as a source of pyrogen.* It is well known that bacteria vary in their pyrogenicity. In the present work it was found that the relative amounts of fever-producing and leukocyte-affecting properties in the vaccines made from two strains of *E. typhosa* were moderately different. In both of these strains, the leukocyte variations were more sensitive than the temperature changes. It is conceivable, however, that other strains of *E. typhosa* or other types of bacteria could affect the temperature more than the leukocytes.

5. *The media in which the organisms are suspended.* The magnitude of the temperature and leukocyte response of the rabbit to typhoid vaccine is dependent upon whether the organisms are suspended in plasma or saline. Ten times the number of organisms were required in a saline suspension to produce a thermogenic and leukocytic response comparable to those obtained with a given number of the same organisms suspended in plasma. The difference between the two menstrua is quantitative except that the onset of fever is always earlier when the organisms are given in plasma. Probey and Pittman

(10) found that only 1/10 as many viable organisms are required to produce a given thermogenic response in rabbits when the organisms are grown in rabbit serum for 2-14 days. It is evident that growth in serum is not a necessary factor for augmentation since it also occurred in our experiments when killed *E. typhosa* organisms were suspended 12-18 hours in homologous plasma.

Summary and conclusions. The effects of intravenously administered typhoid vaccine upon the leukocyte population and the temperature of the rabbit were studied.

The response of the temperature and of the leukocyte count was dependent upon the amount of typhoid vaccine given. Small amounts of the vaccine caused little or no fever, a heterophile leukocytosis, and a mononuclear leukopenia. When larger doses of vaccine were given, the rabbits responded with high fever, an initial heterophile leukopenia followed by a secondary leukocytosis, and a sustained mononuclear leukopenia. Ten times the number of organisms were required in saline suspensions to produce a thermogenic and leukocytic response comparable to that produced when the vaccine was given in homologous plasma. The plasma-typhoid and saline-typhoid reaction differed only in degree except for the earlier onset of fever when the organisms were given in plasma. These experiments on rabbits with typhoid vaccine indicate that when leukocyte changes are used as a test for pyrogens, it is necessary to employ a series of total and differential counts on each animal.

The authors are indebted to P. K. Schork, HMC, USN, and C. H. Gayhart, HMl, USN, for technical assistance; to T. F. Probey, National Institutes of Health, Bethesda, Md., for his helpful suggestions in the preparation of this report; to Dr. H. C. Batson, Army Medical School, Washington, D. C., for furnishing us with strain 58 *E. typhosa;* and to Lt. Commander M. T. Sproul, HW, USN, National Naval Medical School, Bethesda, Md., for her valuable technical advice.

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Received October 10, 1950. P.S.E.B.M., 1950, v75.

The Absorption and Distribution of Labeled Fatty Acids in the Rat.* (18299)

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The immediate distribution and deposition of ingested fatty acids into the lipid fractions of tissues have not been extensively studied. Various investigators(1-7) observed the passage of unsaturated fatty acids into tissues. Cavanagh and Raper(8) found the concentration of deuterium from "deuterated" linseed oil to be greater in the glyceride fatty acids of rat livers and less in the acetone-insoluble phospholipids. Similar technics were used by Sperry *et al.*(9) who showed significant concentrations of deuterium in liver and intestinal fatty acids and much smaller concentrations in depot fat. Using C^{14}-labeled sodium acetate, Pihl and Bloch(10) found that liver neutral fat was regenerated more rapidly than liver phospholipids in contrast to the fat in the mesentery and other tissues in which the isotope concentration was higher in the phospholipid fraction than in the neutral fat.

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* The material presented in this paper is taken from a thesis submitted by John G. Coniglio in partial fulfillment of the requirements for the degree of Doctor of Philosophy from Vanderbilt University and presented in part at the Federation of American Societies for Experimental Biology, April 18, 1950.

† Holder of an A.E.C. predoctoral fellowship.

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At the time this work was begun, pure radioactive fatty acids were not available. We prepared radioactive lipids by injecting C^{14}-labeled sodium acetate into a white rat, extracted the lipid from the carcass and fed this to other rats. These were killed, the lipid extracted from various organs and examined for radioactivity.

Experimental. Carboxyl-labeled acetic acid was prepared from CH_3MgBr and $C^{14}O_2$. 16.56 mg of the sodium salt and 400 mg of sodium pyruvate(11,12) were injected in 2 nearly equal doses. The interval between doses was 20 hours and the rat was decapitated 48 hours after the second dose. The total number of counts injected into the rat was 6.4 x 10^7, and of this amount 1.14 x 10^6 c/m or 1.78% of the administered dose was found in the fatty acids isolated from the tissues studied. The liver and carcass contributed almost all of this activity, only 0.14% coming from the mesentery and intestines. Approximately 90% of the counts administered was found in the expired CO_2. The C^{14}-labeled fatty acids were isolated by alkaline alcohol extraction from an ether solution of the pooled fatty acids obtained from the liver, intestines, mesentery and carcass and were purified by the method described by Armstrong(13). Water-soluble, short chain fatty acids were extracted from the mixture by thorough washing with hot water. Because of the small amount of labeled material available, fractionation of the fatty acids was not attempted. Acetic acid has been shown(11, 14) to serve as a precursor of the higher fatty acids. Because of this and the fact that short chain fatty acids were removed, the material fed presumably consisted mostly of long chain fatty acids.

Feeding experiments. The C^{14} fatty acids were dissolved in olive oil. One ml of the solution was administered by stomach tube to each of 5 young white rats weighing between 100 and 130 g, which had been fasted for 24 hours. The labeled fatty acids received by rats 2 and 3 assayed 671 c/m/mg; those received by rats 4, 5 and 6 assayed 654 c/m/mg. Rats 2, 3 and 4 and rats 5 and 6 were decapitated 5 and 7 hours after feeding, respectively. During the absorption period the animals were kept in individual metabolism cages and water was furnished *ad libitum.* Each cage was contained in an airtight "dry box" furnished with an inlet tube for introducing CO_2-free air and an outlet tube for removing the expired CO_2. Urine and feces were collected and expired CO_2 was collected in traps containing NaOH solution.

Isolation of lipids. The tissues were cut into small pieces and washed with a minimum of physiological saline to remove excess blood. Tissue lipids were extracted from the liver and mesentery by methods similar to those described by Bloor(15) and by Entenman and Chaikoff(16). After the combined ether and alcohol extracts had been evaporated to dryness in a stream of nitrogen and under reduced pressure, the residue was dissolved in petroleum ether and clarified by filtering through fat-free filter paper. The extracts were made to volume with petroleum ether and aliquots taken for the analyses listed below. Free fatty acids in the tissue extracts

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were separated by alkaline alcohol extraction and determined before the lipid extracts were fractionated. Total fatty acids and fatty acids obtained from the various fractions were determined by the method of Kelsey(17). Phospholipids were isolated by precipitation with acetone and $MgCl_2$ as described by Bloor(15,18) and determined by measuring the phosphorus content(19). The acetone-soluble fraction was used for the determination of the activity of the combined fatty acids derived from triglycerides and cholesterol esters and also for the enzymatic hydrolysis of the triglycerides with castor bean lipase(17). The fatty acids thus obtained by the selective hydrolysis of neutral fat were analyzed for activity and compared with the mixture of triglycerides and cholesterol ester fatty acids.

Radioactivity determinations.§ The fatty acids in the fractions listed above were converted to CO_2 by wet combustion, using the combustion fluid of Van Slyke and Folch as prepared by Lindenbaum *et al.*(20) and using the method of the latter with modification to suit our combustion apparatus, as previously described(21). CO_2-free NaOH was used for the absorption of the $C^{14}O_2$. The radioactive carbonate was precipitated as $BaC^{14}O_3$, centrifuged, washed with CO_2-free water, and transferred as a slurry in methyl alcohol to shallow cups. They were dried initially under an infra-red lamp and finally in an electric oven. All samples were counted with a thin window (1.64 mg/cm^2) Geiger-Müller tube. The results were corrected for self absorption, and the counting time for each sample was long enough to insure a reliable error of less than ± 5%. Background was determined before and after each series of samples analyzed and more often when samples of activity very close to background were encountered. Duplicate determinations were done where possible.

Discussion. Satisfactory absorption from the intestine occurred, ranging from 73 to 90% and less than 1% of the C^{14} was expired as CO_2. The greatest C^{14} activity was found in the liver and mesenteric fat. Significant activity was also present in peri-renal and gonadal fat though it could be demonstrated only qualitatively by mounting the fatty acids directly. Feces were excreted by only 2 of the rats during the experimental period. Radioactive carbon was present in both instances. Four rats excreted urine and all samples contained C^{14}. Blood total lipids showed a high activity.

The data from the examination of liver and mesenteric lipids are given in Table I. It should be pointed out that the figures in columns 1-4 represent specific activity, not mgs and are directly related to the counts administered. In each experiment the specific activity of the liver total fatty acids exceeded that of the mesenteric fatty acids. In the liver the fatty acids derived from the neutral fat or acetone-soluble fraction contained the highest concentration of radioactive carbon. The specific activity value observed for the liver total fatty acids was lower than that of the neutral fat fatty acids, a result of the dilution of this very active fraction by the less enriched fatty acids of the phospholipids. The ratio between the concentrations of these 2 fractions ranged narrowly about an average of 2.24. This finding is in agreement with those of other investigators(8,10) and indicates that in the liver a mechanism may be active in keeping the ratio of neutral fat and phospholipid constant during the transport of ingested fatty acids. This situation apparently does not exist in the mesentery where the ratios were variable.

The activity of the free fatty acids and phospholipid fatty acids in the liver was identical in 4 instances indicating a common origin of these fractions. That the free fatty acids do not represent preformed fatty acids but originate from the autolytic decomposi-

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TABLE I. Summary of Radioactivity Data of Lipid Fractions of Liver and Mesentery.

Rat No.	Fatty acids fed (mg)	% absorbed	Specific activity* (× 10²) Liver 1	Liver 2	Liver 3	Liver 4	Liver 4/3	Mesentery 1	Mesentery 2	Mesentery 3	Mesentery 4
2	107	83	2.53	1.21	1.08	3.02	2.79	1.15	1.90	0.79	0.68
3	83	85	1.63	1.11	1.15	2.07	1.80	—	0.92	0.06	—
4	275	73	2.81	2.42	1.90	4.30	2.26	0.17	0.80	0.43	0.17
5	258	90	0.91	0.67	0.65	1.35	2.08	0.23	0.67	0.29	0.24
6	127	81	4.73	2.48	2.34	5.34	2.28	1.34	1.54	0.77	1.04
Mean			2.52	1.58	1.42	3.22	2.24	0.72	1.17	0.47	0.53
Stand. error of mean			±0.65	±0.37	±0.30	±0.72	±0.16	±0.31	±0.24	±0.14	±0.20

$$\text{* Specific activity} = \frac{\text{\% of administered counts}}{\text{mg C}}.$$

1. Total fatty acids; 2. Free fatty acids; 3. Phospholipid fatty acids; 4. Neutral fat fatty acids.

TABLE II. Specific Activity of Liver Fatty Acids Isolated from Triglycerides Alone and from a Mixture of Triglycerides and Cholesterol Esters.

Rat No.	Specific activity (× 10²) Triglyceride fatty acids	Triglyceride + cholesterol ester fatty acids
2	3.02	3.86
3	2.07	2.07
5	1.35	1.54
6	5.34	3.97

tion of the phospholipids of the excised liver is in keeping with Fairbairn's observation(22) of the rapid hydrolysis of phospholipids of extirpated livers of cats and rats. The specific activity of the free fatty acids in the mesentery was always greater than the activity of any other fraction. They must, therefore, be regarded as preformed material, perhaps being deposited as soaps from the chyle.

In Table II a comparison is made of the concentration of C^{14} in the fatty acids of the triglycerides and of the fraction containing both triglycerides and cholesterol esters. Generally speaking, the activity of cholesterol esters was equal to or greater than the activity of the glycerol esters, an indication of the importance of cholesterol in the metabolism of ingested fatty acids.

Summary. Biosynthesized lipids labeled with C^{14} was fed to rats and the lipids from various organs examined for radioactivity. The importance of the observations in studies of lipid metabolism was pointed out.

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Received October 11, 1950. P.S.E.B.M., 1950, v75.

Effect of Leucocytes and Bacteria on Glucose Content of the Cerebrospinal Fluid in Meningitis. (18300)

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The subnormal level of glucose in the cerebrospinal fluid of patients with bacterial meningitis is frequently of diagnostic value in differentiating bacterial from aseptic meningitis(1). Reasons for the reduced concentration of glucose in bacterial meningitis are not understood although it is stated that the main factor is utilization of glucose by bacteria growing in the fluid(1). On the other hand, the concentration of glucose in aseptic

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meningitis is said to be normal because the leucocytes do not metabolize it(1). The object of the present investigation was to assess the importance of bacterial growth and leucocytic metabolism as factors tending to lower levels of glucose in the cerebrospinal fluid. It has been found that leucocytes and pneumococci utilize glucose in the cerebrospinal fluids of dogs but the data suggest that these factors are not sufficient in themselves to account for the low levels of glucose in cerebrospinal fluid of bacterial meningitis.

Methods. Tests were carried out with the cerebrospinal fluids of dogs rather than human beings because the production of aseptic meningitis by intracisternal injection of irritating substances made it possible to exclude more surely the participation of bacteria. The dogs were anaesthetized by intravenous nembutal and subjected to cisternal puncture with a 20 gauge needle. After withdrawal of 3 to 5 ml of cerebrospinal fluid, equal amounts of sterile normal rabbit serum or distilled water containing 5000 units of penicillin were administered intracisternally. Bacterial meningitis was produced similarly by intracisternal injection of 0.5 to 1 ml of a 12 to 18 hour culture of virulent Type III pneumococci. Further cisternal punctures were done after varying periods for examination and tests of fluid. The number of polymorphonuclear leucocytes per cubic millimeter of cerebrospinal fluid was determined in the usual manner(1). Absence of bacteria from fluids of dogs with aseptic meningitis was confirmed by aerobic and anaerobic cultures on rabbit blood agar plates(2). Pneumococci to be placed in normal cerebrospinal fluid were grown for 4 hours in beef infusion broth containing 10% normal sheep serum and 0.2% glucose. Small amounts of young cultures prepared in this manner were transferred by pipette to the cerebrospinal fluids, and numbers of pneumococci in the fluids were determined at hourly intervals. Bacterial content was determined by removing 0.5 ml of the fluid, making serial ten-fold dilutions in broth, followed by colony counts of poured blood agar plates. Concentrations of glucose were determined by standard methods(3,4). For measurement of the rate of consumption of glucose *in vitro,* fluids were shaken constantly at 37°C in a Warburg water bath as recommended by Barron and Harrop(5).

Results. Concentrations of glucose in the cerebrospinal fluids of dogs with aseptic meningitis are shown in Table I and were found to be essentially normal as judged by standards in human beings(1). Similar determina-

TABLE I. Glucose Levels in Cerebrospinal Fluids of Dogs with Aseptic and Pneumococcal Meningitis.

Exp. No.*	Type of meningitis	Leucocytes per cu mm	Conc. of glucose (mg per 100 ml)
1	Aseptic	2227	80
8	,,	4284	61
19	,,	30000	50
23	,,	13000	53
24	Pneumococcal	25000	28
28	,,	8000	17
29	,,	30000	22
31	,,	11520	23†

* To conserve space, only representative experiments are presented.

† Bacterial count of this specimen revealed 250000 pneumococci per ml of fluid.

TABLE II. Consumption of Glucose *In Vitro* by Cerebrospinal Fluids of Dogs with Aseptic Meningitis.

Exp. No.	Leucocytes per cu mm	Conc. of glucose: Before incubation	Conc. of glucose: After incubation	Consumption of glucose per hr (mg per 100 ml)
1	1305	72	61	11
2	3000	78	70	8
6	6400	45	47	0
5	13000	48	32	16
7	15000	74	63	11
9	20700	53	42	11
12	25920	66	49	17
3	30000	50	26	24
11	37000	49	36	13
10	43000	60	33	27
4	57600	128	98	30

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TABLE III. Consumption of Glucose *In Vitro* by Pneumococci Growing in Normal Cerebrospinal Fluid.

Exp. No.	Time (hr)	No. of pneumococci	Conc. of glucose (mg per 100 ml)	Consumption of glucose per hr (mg per 100 ml)
1	0	6×10^7	80	3
	1	15×10^7	77	26
	2	95×10^7	51	48
	3	15×10^8	3	
	4	15×10^8	0	
2*	1	6.7×10^6	85	3
	2	79×10^6	82	8
	3	34×10^7	74	20
	4	9×10^8	54	23
	5	2×10^8	31	

* For this experiment, glassware was cleaned by boiling in equal parts of concentrated nitric acid and distilled water.

tions in pneumococcal meningitis are also recorded in Table I and are lower than normal by the same standards. In Table II, it may be seen that definite amounts of glucose were consumed by fluids of dogs with aseptic meningitis and that the rate of utilization appears to be more rapid in fluids with higher cell counts. Table III shows the consumption of glucose *in vitro* by pneumococci growing in normal cerebrospinal fluid and it may be noted that only with extremely large numbers of pneumococci growing rapidly did the rate of consumption of glucose approximate that utilized by the leucocytes in fluid of aseptic meningitis.* In control experiments not recorded in the tables, prolonged incubation of normal cerebrospinal fluids did not result in diminished concentrations of glucose.

Discussion. Measurements of glucose before and after incubation of cerebrospinal fluids of dogs with aseptic meningitis have demonstrated that glucose is utilized by such fluids. Although two previous studies(1,6) report failure to detect consumption of glucose under somewhat similar conditions, several others(7-11) have presented qualitative experiments indicating that cerebrospinal fluid containing leucocytes but no bacteria is capable of using glucose. Consumption of glucose by these fluids must be due to the leucocytes and is not surprising in view of the considerable published evidence(5,12-17) showing that leucocytes from other sources metabolize glucose *in vitro*.

Normal levels of glucose are known to occur in aseptic meningitis of human beings(1) and this fact has been confirmed for dogs in the present experiments. Normal concentrations of glucose under these circumstances might not be expected in view of the fact that fluids from such dogs were shown to utilize glucose. However, an adequate explanation for this discrepancy would appear to be that glucose of the cerebrospinal fluid is replenished more rapidly by the body than it is utilized by the leucocytes.

It is important to note that *in vitro* tremendous numbers of rapidly growing pneumococci were necessary in order to consume glucose in cerebrospinal fluid as rapidly as the leucocytes in the fluids from dogs with aseptic meningitis. Since the utilization of glucose by less than 150 million pneumococci per ml was so low that it could hardly be measured it is probable that the consumption of glucose by the much smaller numbers of pneumococci usually present in meningitis would be so small as to be insignificant.† Previous workers(7,8,10,11) have added bacteria to cerebrospinal fluid and have shown consumption of glucose but have not attempted to measure rates of utilization of glucose under these conditions. On the other hand, studies of the consumption of glucose by bacteria in culture media have shown even lower rates than were found in the present experiments (18-20).

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* In the single instance where utilization of glucose by the bacteria was greater than that of leucocytes, the number of pneumococci was particularly large and the rate of growth especially rapid.

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Inasmuch as normal mechanisms of replenishment appear to suffice to maintain glucose levels in the cerebrospinal fluid when leucocytes are present without bacteria, and since the numbers of bacteria in the cerebrospinal fluid in meningitis are usually much smaller than were used in the *in vitro* tests, it seems likely that additional factors must be operating to cause the lowered levels of glucose in the cerebrospinal fluid of bacterial meningitis. Especially, does it seem probable that other factors than utilization of glucose by leucocytes and bacteria are present in tuberculous meningitis. The numbers of leucocytes and bacteria are not great enough in this disease to overcome normal mechanisms of replenishment, particularly since there is evidence that lymphocytes consume less glucose than granulocytes(5,16) and that tubercle bacilli grow slowly with a low rate of utilization of glucose(21).

Other possibilities have been suggested as mechanisms for low levels of glucose in the cerebrospinal fluid in bacterial meningitis. For example, there may be a change in the permeability of the blood-cerebrospinal fluid barrier(9,11,22-24); or damage to meninges and choroid plexus may increase the glycolytic rate of these tissues(7,9). To support these theories there is as yet no direct experimental evidence.

Summary. 1. Levels of glucose in cerebrospinal fluids of dogs with aseptic meningitis were normal, while incubation *in vitro* of such fluids resulted in consumption of glucose. Evidently, glucose is transported into the cerebrospinal fluid *in vivo* more rapidly than it is utilized by the leucocytes under these conditions. 2. Very large numbers of pneumococci growing rapidly in normal cerebrospinal fluid were needed in order to consume glucose at rates approximating those at which glucose is utilized *in vitro* by fluids of dogs with aseptic meningitis. 3. Consumption of glucose by leucocytes and bacteria does not appear to account for lowered levels of glucose in bacterial meningitis.

We are indebted to Dr. W. B. Wood, Jr., and Dr. C. V. Moore for advice and encouragement.

Thanks are due to Dr. J. O'Leary and Dr. Ethel Ronzoni for additional facilities needed for completion of the work.

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† The number of pneumococci in the cerebrospinal fluid of a dog with acute meningitis was found to be 250,000 per ml (Table I, Experiment 31).

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Received October 13, 1950. P.S.E.B.M., 1950. v75.

Effect of Aspirin Upon Glycosuria of the Partially Depancreatized Rat. (18301)

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The data of this report show that large doses of aspirin (acetylsalicylic acid) suppress the glycosuria of the partially depancreatized rat.

Methods. Male rats of the Sprague-Dawley strain were subjected to partial pancreatectomy(1) at a weight of approximately 300 g. Following recovery from the operation, the animals were placed in metabolism cages and were force-fed a medium carbohydrate diet (2) by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with toluene. Urine glucose was determined by the method of Benedict(3). Aspirin was ground into a fine suspension in peanut oil at a concentration of 100 mg per cc. It was administered by subcutaneous injection in divided doses each morning and late afternoon.

Results. Seven mildly diabetic rats were observed during a control period of several weeks. During the daily administration of 40, 80, and 160 mg of aspirin for one week, at each dose level, there was a marked decrease in the glycosuria in each animal at each dose level together with a concomitant gain in weight. In each rat, the quantity of reducing substances in the urine was depressed below 200 mg on one or more days during treatment with aspirin. When the injections of aspirin were stopped, there was an exacerbation of the glycosuria to levels much higher than were observed during the initial control period, and there was an accompanying loss of weight. The data are summarized in Fig. 1.

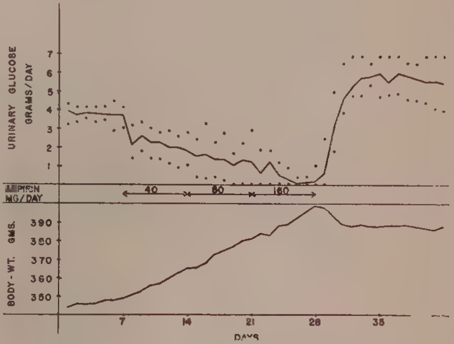

FIG. 1.

Effect of aspirin upon the urinary glucose and body weight of 7 partially depancreatized force-fed rats. Averages and range.

Discussion. In this laboratory, we search for compounds which modify experimental diabetes. Aspirin and cortisone each suppress the symptoms of rheumatoid arthritis. Cortisone causes exacerbation of diabetes in mildly diabetic animals. This was the basis for speculating about the effect of aspirin upon the level of glycosuria in such animals. The effect of aspirin in ameliorating the glycosuria is unlike the effect of cortisone. The mechanism of its action and its possible relationship to the function of the adrenal cortex are unknown. It is interesting to note that the effect of aspirin upon glycosuria is as marked as the ameliorating effect of adrenalectomy in our experience(4).

The possibility that aspirin may conjugate with glucuronic acid and be excreted in a form which would not be detected by tests for reducing substances has been pointed out to us. Since the possible conjugation would be molecule per molecule, the amount of aspirin administered could not mask the excretion of more than an equivalent weight of reducing substances. This would represent a very small fraction of the actual decrease in urinary reducing substances during treatment with aspirin.

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The amount of aspirin required to ameliorate the glycosuria of the rat is very large, and there is no reason to expect that this drug would be of any therapeutic value in clinical diabetes.

Summary. Aspirin was administered to 7 mildly diabetic rats which were force-fed a medium carbohydrate diet. The dose was 40, 80, and 160 mg of the drug per day. A marked amelioration of the glycosuria together with a gain in weight was noted in each rat. When the drug was withdrawn, there was exacerbation of the glycosuria to significantly higher levels than were noted during the pre-injection period, and there was an accompanying loss of weight.

Addendum. Since this manuscript was submitted for publication a review (Gross, M. and L. A. Greenberg, Hillhouse Press, New Haven, Conn., 1948. pp 108-9) of the effects of salicylates upon carbohydrate metabolism has been noted. Salicylates were shown to have an ameliorating effect upon diabetes mellitus in man as early as 1875, but large and frequently toxic amounts were required and the use of salicylates for this purpose has long since been discontinued. The nature of the action of salicylates upon carbohydrate metabolism remains as an interesting problem.

Received October 13, 1950. P.S.E.B.M., 1950, v75.

A New Criterion for Anti-tumor Activity of Tissue Extracts Using Crocker Sarcoma No. 180 in Mice.* (18302)

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When Crocker Sarcoma No. 180[†] is implanted into CFW male mice (Carworth Farms), a loss in weight of the animals invariably occurs on the 7th or 8th day following implantation at a time when the tumors are in rapid growth. One is tempted to regard this loss in weight as concurrent with the development of a cachectic state, somewhat analogous to what occurs in advanced human cancer cases. Apparently most of the transplantable tumors fail to show the same behavior. We have investigated in this respect, the Walker Carcinosarcoma 256 transplanted into Sherman, Wistar and Sprague-Dawley rats, Mammary Adenocarcinoma E.O. 771 transplanted into C-57 female mice (Rockland Farms), a spontaneous mammary tumor (Cystoadenocarcinoma) found in Swiss Webster female mice (Rockland Farms) and in fewer instances in the Rous Sarcoma of chickens. All these tumor strains showed either no loss in weight, due to the tumor, or this occurred only in some cases. It seems, however, possible that some other tumors may be found, in which this phenomenon can be demonstrated.

We have used the above tumors for routine assay of extracts obtained from various organs in assaying the potency of tumor-inhibiting and tumor growth-stimulating activity. Some 130 experiments were performed in the last 3 years in this direction. When we used such active fractions in the first mentioned tumor (Crocker Sarcoma No. 180) we found invariably, that while control animals showed a loss in body weight, tumor-growth-inhibiting fractions (in doses 5-10 mg/mouse, total dose 40-80 mg) caused body weight increases, while tumor-stimulating fractions showed even larger weight losses than the controls (Fig. 1) Normal controls, not bearing the tumor, gain on an average 10 g during the experimental

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* We wish to thank the officers of the U. S. Vitamin Corp., H. B. Burns, President, for the grant they have extended to us.

[†] The spontaneous tumor used was obtained from Rockland Farms, Rous Sarcoma was kindly supplied to us by Dr. A. Claude, of the Rockefeller Institute, N. Y., all the other tumors used in this work were most generously made available by Dr. K. Sugiura, Sloan-Kettering Institute, N. Y., to whom our sincere thanks are due.

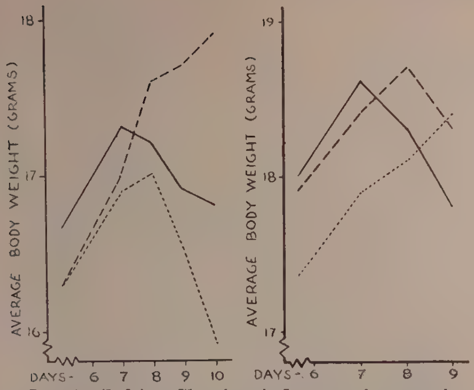

Fig. 1. (left). Showing influence of tumor implantation on controls (15 mice) and treated mice (15 each). Controls, ————; tumor-inhibiting fraction, – – – – (39.8% tumor wt decrease); tumor-stimulating fraction, ... (38.7% tumor wt increase).

Fig. 2. (right). Controls, ————; tumor-inhibiting fraction, – – – –, single dose (20.0% tumor wt reduction); tumor-inhibiting fraction, ..., double dose (53.0% tumor wt reduction).

period chosen. They reach a plateau at 24 g body weight.

By using single and double doses of the same material, a more accentuated response is obtained (Fig. 2). It is obvious that only fractions or substances with low toxicity can be used in this reaction. In cases in which we are dealing with a mixture of inhibiting and stimulating fractions the above reaction does not show, unless the 2 fractions are separated from each other. The evidence of such behavior can be obtained by using single and double doses of the same mixture (Fig. 2). A histological study‡ of the tumors revealed in the controls an invading tumor into the striated muscle, with rounded polyhedral and spindle shaped patterns, with widespread necrosis and reaction changes to necrosis. Treated animals (those on double dose were more accentuated) showed surprisingly more mitotic figures and more anaplasia. It may be mentioned that Cornman (1) reports, that the factors influencing mitosis may be different from those which cause reduction in tumor size.

Summary. Mice implanted with Crocker Sarcoma No. 180 invariably lose weight suddenly after 7-8 days. When injected with tumor-inhibiting fractions they continue to gain weight, with tumor-stimulating fractions the opposite effect takes place. Definite evidence of the two effects have been demonstrated.

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‡ The histological work was done by Dr. A. Angrist, pathologist to the Queens General Hospital, N. Y., through the kind intervention of Dr. J. M. Brown. We wish to extend our thanks to both these gentlemen.

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Received October 18, 1950. P.S.E.B.M., 1950, v75.

Effect of Sugars on Erythrocytes Preserved at 0° to –3°C.* (18303)

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From the John S. Sharpe Research Foundation and the Laboratory of Clinical Pathology of the Bryn Mawr Hospital, Bryn Mawr, Pa.

Proper amounts of dextrose are known to improve preservation of stored erythrocytes. The use of other sugars has been reported by several workers(1). This report deals with the effect of certain sugars on the osmotic fragility of erythrocytes maintained at temperatures between 0°C and –3°C and the result of transfusions of blood thus preserved in humans. The fragility studies(2) were carried out for the purpose of general screen-

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* This work was, in part, carried out with the support of the Army Medical Research and Development Board under contract with the Bryn Mawr Hospital

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ing; erythrocytes showing diminished resistance to hypotonic salt solution invariably showed poor survival when transfused. The *in vivo* studies included post-transfusion variations in the blood volume, erythrocyte count, hematocrit, bilirubin level, urobilinogen output and studies of survival by the non-agglutinable cell count. In some instances it has been necessary to choose, for the time being, an arbitrary value for some of the experimental conditions (such as pH, volume of resuspension fluid, etc.) on the basis of work previously done. The complex interrelationship of the various elements may require readjustment of many of the values here reported. Therefore, none of the data obtained so far can be considered as final. This preliminary report appears desirable in view of the fact that the *in vitro* and *in vivo* studies thus far carried out strongly suggest that, with proper technic, red cells can be preserved without hemolysis for periods of time much longer than now obtained with any of the acid-citrate-dextrose solutions and that such cells remain in circulation, when transfused, at least as long as the red cells of whole blood collected in acid-citrate-dextrose solutions and stored for less than 21 days.

Material and technic. Erythrocytes were used as whole acid-citrate-dextrose (A.C.D.) blood(3), as acid citrated packed erythrocytes, and as acid citrated packed cells resuspended in a 4% globin solution. The packed cells were obtained by bleeding in a cooled sodium citrate solution prepared as follows: Trisodium citrate dihydrate g 2.1; citric acid monohydrate 0.66 g; water to 100 ml. Seventy-five ml of this solution were used for each 500 ml of blood. Within 12 hours of collection, after storage at 1°C, the blood was centrifuged and 80% to 85% of the plasma removed. To the red cell residue, solutions of the various sugars with or without globin were added. All the concentrations noted are weight to volume of the suspension medium. The pH of the fresh A.C.D. blood, in all instances, was between 7 and 7.2. The cells were maintained in rubber stoppered test tubes or cylindrical bottles in aliquots of 10 to 60 ml at –3°C (±0.2°C) in the liquid state(4) or at higher temperatures up to +4°C. When the blood was intended for transfusion into humans, it was collected and preserved in the standard 500 ml bottles. Maltose, sucrose and lactose were tested in concentrations of 1 to 12%. In all instances the solutions of sugars were sterilized by Seitz filtration.

Experimental results. Early in the experimental work acid-citrated packed erythrocytes were found to deteriorate faster than erythrocytes preserved as whole blood or erythrocytes resuspended in a volume of 4% globin equivalent to the amount of plasma removed. Maltose, although exerting a favorable effect on preservation, was found to give inconsistent results.

The effect of temperature of storage was studied in relation to addition of sucrose and lactose. It was found that preservation was optimal, under the experimental conditions, with temperatures between 0°C and –3°C. Addition of sugars to blood preserved at +4°C improves preservation, but to a lesser degree.

When erythrocytes are preserved with the disaccharides, shrinkage of varying degree occurs, along with a striking change in the osmotic fragility curve (Fig. 1). The amount of shrinkage, however, does not necessarily have a direct relationship to preservation. Thus lactose, which produces in the same concentration less shrinkage than sucrose, provides a better preservation (Fig. 4).

It is notable that the results of the osmotic fragility are the same whether the cells are transferred directly from the suspension medium to the hypotonic salt solutions or whether they are previously swollen back to their normal size by immersion in normal plasma.

The optimal concentration of lactose and sucrose has not been determined but, with whole blood, good results are obtained with

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4. Strumia, M. M., *Science*, 1949, v110, 398.

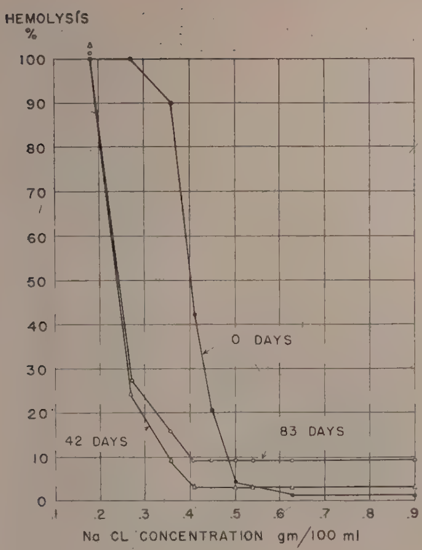

FIG. I

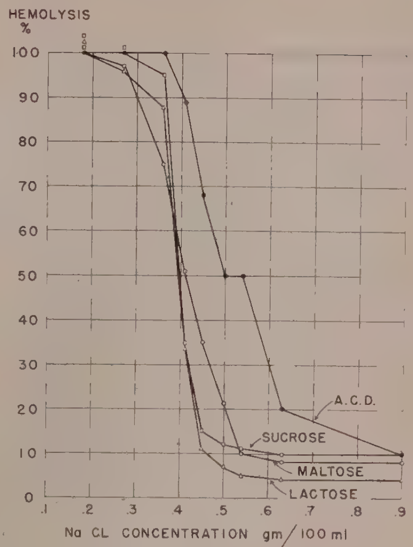

FIG 2

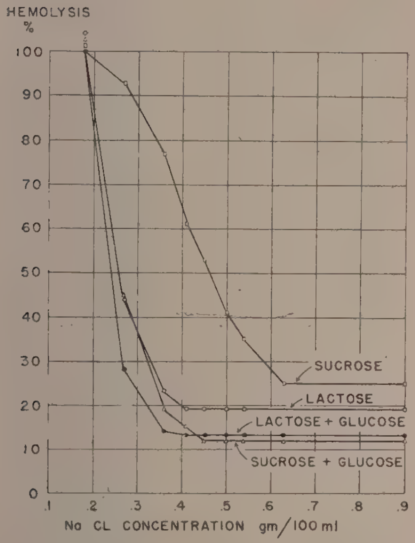

FIG 3

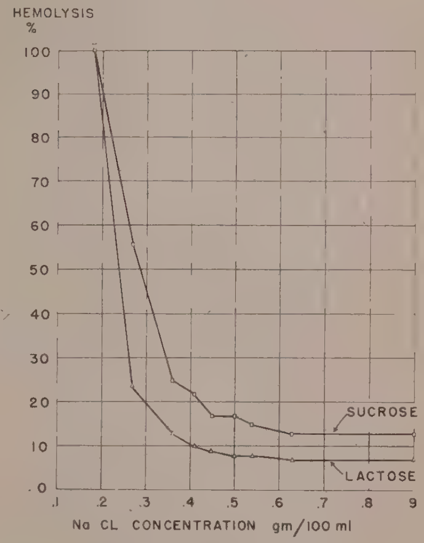

FIG. 4

Fig. 1. Changes in osmotic resistance of erythrocytes resuspended in: Globin 4%, lactose 5%, dextrose 250 mg %—storage at ±0°C.

Fig. 2. Effect of addition of 5% of sugars on the osmotic resistance of erythrocytes preserved at —3°C for 35 days (as packed red cells).

Fig. 3. Effect of glucose (250 mg/100 ml of plasma) on the osmotic fragility of erythrocytes when added to whole blood containing 5% sucrose or lactose. Blood preserved at —3°C for 49 days.

Fig. 4. Effect of sucrose and lactose in a 5% concentration on the osmotic resistance of erythrocytes preserved at —3°C for 98 days. (As cells resuspended in globin).

concentrations of 3% to 7%. When erythrocytes resuspended in 4% globin solution are used, somewhat higher concentrations of sugars appear to exert a better effect.

Fig. 2 shows the effect of sugars in a concentration of 5% on the fragility curve of whole acid-citrated blood stored, without addition of glucose, for a period of 35 days at −3°C. The fragility curve of a standard ACD blood, stored under identical conditions, is shown for comparison. Fig. 3 shows the effect of the addition of 250 mg of glucose/100 ml of plasma containing 5% sucrose or lactose. The added glucose shows a definitely beneficial effect on the preservation of blood. The absence of glucose is felt more when sucrose is used than when lactose is used. Glucose, in concentrations comparable to those of sucrose and lactose, produced gross hemolysis within one week of storage.

A number of experiments were carried out to test the relative value of sucrose and lactose when glucose is added (250 mg/100 ml of suspension medium). A difference is noted only when the experiment is carried over a period of 1½ to 3 months or longer. In order to carry the preservation over the three months' period it is necessary to remove plasma and replace it with an equal amount of a protein solution. A 4% solution of globin has been found very useful for this purpose. Fig. 4 gives the result of one of the experiments carried out for 98 days.

Preliminary studies with decalcified blood, using either a modified phenol-formaldehyde sulfonic type of resin ("Amberlite")* or a sulphonated aromatic hydrocarbon polymer ("Nalcite")† show that under the experimental conditions preservation of erythrocytes with or without sugar is not as good as when acid-citrated blood is used. A comparison was also carried out between wettable and non-wettable apparatus, using plastic tubing, silicone coated glassware and stainless steel cannulas coated with dicocodimethyl ammonium chloride ("Arquad 2C").‡ The extent of this experimental work is not enough to justify any conclusion but, so far, no difference was found between the two types of apparatus on the preservation of erythrocytes.

Experiments in vivo. In all, 64 transfusions of about 500 ml of blood have been given to humans, using blood preserved with the aid of sugars. In 36 instances, lactose was used and in 28 sucrose. The concentration of sugars has been between 3% and 5%. To all but 3 specimens dextrose was added in concentration of 250 mg/100 ml. Thirty-four of these transfusions were made with

TABLE I. Variations in the Hematocrit Following 50 Consecutive Transfusions of 500 ml of Whole Blood or a Similar Amount of Resuspended Erythrocytes.

Whole blood or resuspended erythrocytes stored at 0° to −3°C with the aid of sugars and globin solution		Whole ACD blood stored at +4°C	
Days of preservation	Hematocrit variation	Days of preservation	Hematocrit variation
21	4.0	2	1.3
21	4.1	5	3.1
21	2.6	6	2.1
21	3.0	11	3.9
23	2.1	15	—0.5
23	0.5	15	3.0
24	0.9	16	1.8
25	2.4	16	0.4
26	0.5	17	3.3
26	0.6	17	2.2
28	2.0	17	4.0
31	3.3	17	2.2
32	4.1	17	2.5
33	1.2	17	3.4
35	2.8	18	6.3
35	1.7	18	2.9
35	1.7	18	2.1
40	0.1	19	—0.1
40	0.4	19	2.7
41	0.3	20	—4.0
41	1.2	20	2.1
41	1.5	20	2.3
42	0.8	20	5.2
42	3.9	22	2.1
47	5.0	23	0.0
Avg 31.7	2.02	14.2	2.08

There is a small loss of erythrocytes in the preparation of resuspended cells. An exact measure of this loss is not available because the technic has so far been somewhat variable. In addition, the subjects receiving blood preserved at 0° to —3°C have generally been patients suffering from terminal forms of carcinoma or leukemia when determination of hematocrit as a measure of survival of red cells is more likely affected by hemorrhage or excessive hemolysis. The relatively small gains in both series are due to the fact that the post transfusion hematocrit was taken 24 hr after transfusion at a time when most of the transfused plasma had been retained, reducing the percentual rise of erythrocytes.

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* Rohm and Haas Co., Philadelphia, Pa.

† National Aluminate Co., Chicago, Ill.

‡ Armour Co., Chicago, Ill.

whole blood, 30 with erythrocytes resuspended in 4% globin containing sucrose or lactose. The period of preservation varied from 14 to 47 days and the temperature of storage varied from –3°C to +4°C. In all instances the blood was maintained in the liquid state except for one specimen which was maintained in the solid state at –3°C (4). In this series there were 2 reactions, one due to pyrogens and one to Rh incompatibility in a known Rh negative patient receiving repeated injections of Rh positive blood. Neither reaction can be attributed to the specific method of blood preservation used. In the majority of cases the changes in the hematocrit and hemoglobin concentrations, the variations in the serum bilirubin content and urobilinogen output were those expected from the transfusion of ACD blood preserved up to 21 days. (Table I.) Blood volume studies were carried out in a number of occasions and in 5 patients, survival studies by the non-agglutinable cell count were carried out using the technic suggested by Young, Platzer and Rafferty(5). One patient suffering from a hypoplastic anemia received two 500 ml lots of blood preserved with 5% sucrose at –3°C for 14 and 21 days respectively, and two 500 ml of lots of blood preserved with 5% sucrose at 0°C for periods of 31 and 40 days. Evidence from studies of the blood volume, hematocrit, hemoglobin concentration, serum bilirubin and non-agglutinable cell count showed good preservation of the transfused cells.

One patient recovering from coronary thrombosis received 500 ml of blood preserved at –3°C with 5% sucrose for 25 days. The same studies as carried out in the preceding case indicated good retention of the transfused cells. These findings are contrary to those reported by Mollison and Young(6). The lower temperature of preservation and the addition of glucose is possibly responsible for this difference. From the *in vivo* studies, packed erythrocytes resuspended in 4% globin with 5% lactose and stored at 0° to –3°C, gave the most encouraging results. For this reason, blood stored in this manner was used in detailed experiments using 3 healthy recipients. These 3 recipients were normal, young male volunteers who were bled approximately 500 ml and then transfused an amount of stored blood containing the same quantity of red cells removed. The period of preservation of the blood used was 30, 32 and 39 days respectively. In all instances 80% to 85% of the plasma was replaced by 4% globin solution containing 5% lactose and 250 mg/100 ml of dextrose. Preservation was carried out at 0°C (±0.2°C).

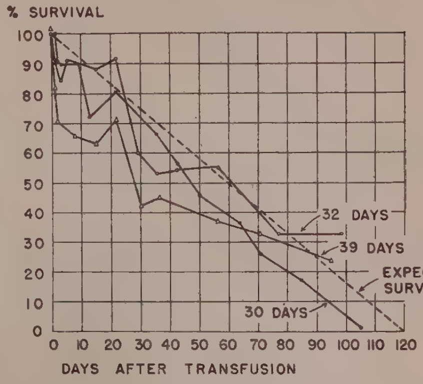

Fig. 5.

Survival of transfused erythrocytes preserved with 5% lactose at 0°C with the non-agglutinable cell count. 100% represents the ideal value on non-agglutinable cells if no transfused cells were lost.

Fig. 5 shows the survival of transfused cells by the non-agglutinable cell count technic. Erythrocyte and plasma volume studies were carried out before and 48 hours after the transfusion by the dye method and air turbine hematocrit; the non-agglutinable cell count was determined repeatedly prior to transfusion; the volume of cells to be transfused was measured and from these 3 figures the expected 100% survival was calculated as shown in the following example:

Red cell volume (avg of 2 determinations) = 2602 ml

Volume of transfused red cells 138 ml (30 days old)

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5. Young, *et al., J. Lab. and Clin. Med.*, 1947, v32, 49.

6. Mollison, P. L., and Young, I. M., *Brit. Med. J.*, 1941, v2, 797.

Percentage of non-agglutinable cells before transfusion 1%

$$100\%\ \text{survival} = 1 + \frac{13800}{2602} = 5.3\%\ \text{non-agglutinable cells.}$$

As seen from Fig. 5, the survival was that which might be expected from fresh blood with a full life expectancy.

Summary. Sucrose and lactose exert a preserving effect upon erythrocytes when stored at 0°C to –3°C. Sixty-four transfusions of blood thus stored for periods of time up to 47 days have shown good survival of the transfused cells. In the experiments *in vivo* the concentration of sugars varied from 3 to 5%. The experimental conditions were not always necessarily optimal for red cell preservation, and therefore the limits of storage are not maximal and will most likely be extended in future work. No attempt has been made to study the mechanism of action of the sugars on the preservation of red cells.

Received October 18, 1950. P.S.E.B.M., 1950, v75.

Fat Emulsions for Oral Nutrition. II. Failure of Phosphatide, Tween 80, or Choline to Influence Fat Absorption.* (18304)

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This laboratory has studied the absorption of fat from fat emulsions given orally, and has investigated the effect of emulsification of fat on its intestinal absorption, using the rat as the experimental animal(1). It was felt that studies of this type would be of value as preliminary observations toward the use of orally administered fat emulsions in humans with normal and abnormal digestive systems. It was found that gelatine-stabilized emulsions of oil in water were unstable in the rat stomach, but were stable when instilled in the ligated rat intestine. The oil in such emulsions was absorbed more rapidly than when present in the same unemulsified systems. This increased rate of absorption was related to the particle size of the emulsions. Other workers(3) have reported that relatively large amounts of lecithin (16.7% in relation to the fat) increased the absorption of fat given orally to the rat in experiments using approximately twice the amount of fat this laboratory used. Tidwell(4), using 20% lecithin in relation to physiological amounts of orally fed fat, found an increase in the chylomicronemia of rats over controls. It has also been reported(5) that lecithin increased the blood fat levels in normal human subjects and in a small series of idiopathic sprue patients. Tween 80 (polyoxyethylene sorbitan monooleate), a synthetic emulsifier, has been stated to reduce the fecal fat excretion in patients with steatorrhea(6). Irwin *et al.*(7) have investigated the effect of the addition of various substances to fat meals upon fat absorption in the rat and found that they had little or no positive effect. It was also reported(8) that the physical condition of the animal determined

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* Supported in part by grants-in-aid from the National Dairy Council, Chicago, Ill.; the Upjohn Company, Kalamazoo, Mich.; the Nutrition Foundation, New York City; and the Cancer Research Grants Branch, National Cancer Institute, Bethesda, Md.

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2. Geyer, R. P., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J., *J. Lab. and Clin. Med.*, 1948, v33, 163.

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5. Adlersberg, D., and Sobotka, H., *J. Nutrition*, 1943, v25, 255.

6. Jones, C. M., Culver, P. J., Drummey, G. D., and Ryan, A. E., *Ann. Int. Med.*, 1948, v29, 1.

whether orally administered fat would be normally absorbed. Frazer(9) has noted that choline added to a fat meal caused a finer subdivision of the intracellular fat particles in the intestinal epithelial cells and increased the concentration of fat in the areolar tissue of the villus of the rat. Tidwell(4) found that choline deficiency diminished the blood chylomicrons after fat meals.

The present study was undertaken to investigate the absorption of fat in the presence of amounts of emulsifiers greatly in excess of that needed to give good emulsions by mechanical means, both after oral administration of the fat and emulsifiers and after their instillation in the intestinal loop ligated below the pancreas. The role played by choline in fat absorption from the intestine was also studied.

Method. Oral feeding experiments. Albino male rats of the Charles River strain weighing 150 to 250 g were used. They were maintained on a stock diet of Krunchon† and were fasted 48 hours before all experimental procedures. All oral preparations were administered from a calibrated syringe via a stomach tube after light ether anesthesia of 30 to 60 seconds duration. Four hours after intubation, the rats were sacrificed with ether and the total nonsaponifiable and saponifiable lipids remaining in the gastro-intestinal tract were recovered in a manner previously described(1)‡. The preparations fed were as follows: a. 0.45 cc corn oil with 1.5 cc of 6% purified soybean phosphatide(2) in water (0.090 g of phosphatide), b. 0.45 cc corn oil and 1.5 cc of 6% Tween 80 in water (0.090 g of Tween 80). The choline deficient rats were produced by placing the normal animals for one month on a diet(10) consisting of 10% casein, 20% lard, 64% dextrose, 2% cod liver oil, 4% salt mixture(11), plus supplements of 1-cystine, thiamine, riboflavin, pyridoxine, and calcium pantothenate, and were fasted for 48 hours before use. They were fed 0.45 cc of corn oil and treated in similar fashion. Liver sections were taken from each at the time of sacrifice and were studied histologically for the presence of fatty infiltration.

Intestinal instillation experiments. Charles River rats fasted for 48 hours were anesthetized with ether and the proximal jejunum was ligated below all pancreatic tissue and a ligature was also placed at the ileocecal junction. The various preparations used were instilled into the intestinal lumen through a small incision below the first ligature via a calibrated syringe and an 8 cm length of ureteral catheter. Another ligature was tied immediately below the incision and the abdomen was closed. This entire procedure took approximately 10 minutes, and the rats recovered from the anesthesia within 15 minutes of the induction of anesthesia. The rats were sacrificed with ether 4 hours after the instillation, and the contents of the ligated intestine were analyzed for total lipids. The preparations instilled were as follows: a. 0.31 cc corn oil with 1.0 cc of 6% purified soybean phosphatide in water (0.060 g of phosphatide), b. 0.31 cc corn oil with 1.0 cc of 6% Tween 80 in water (0.060 g of Tween 80), c. 0.31 cc of corn oil with 1.0 cc of 6% choline

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7. Irwin, M. H., Weber, J., and Steenbock, H., *J. Nutrition,* 1936, v12, 365.

8. Irwin, M. H., Steenbock, H., and Kemmerer, A. R., *J. Nutrition,* 1936, v12, 357.

9. Frazer, A. C., *Nature,* 1946, v157, 414.

† Generously supplied by Gaines Division, General Foods, Kankakee, Ill.

‡ It has been recently suggested to the authors that the alcohol-ether solution used to flush the gastrointestinal tract of its contents would extract out interacellular fat as well. Water flushes had been used in our preliminary work but discarded as unsatisfactory because of the very poor recovery obtained (only 60 to 80% from rats intubated with known amounts of oil and killed immediately afterward). Sudan stained sections of water flushed intestines of rats sacrificed 4 hours after the stomach intubation of 0.45 cc of corn oil demonstrated that free intralumenal oil was left behind on the mucosal surface. Similar examinations of alcohol-ether washed sections demonstrated no free oil, with only slight loss of intracellular fat from the epithelial cells apparent.

10. György, P., *Am. J. Clin. Path.,* 1944, v14, 67.

11. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.,* 1941, v138, 459.

TABLE I. Recovery of Oil Preparations in Rats Sacrificed Four Hours After Oral Feeding. (6 rats in each series).

Preparation	State of animal	g lipid admin.	Mean g lipid recovered: Stomach	Mean g lipid recovered: Intestine	Mean g lipid recovered: Cecum	Mean total g recovered	Mean g absorbed	Mean % absorbed ± S.D. of M	Stand. error of mean in %
Corn oil*	Normal	.350	.043	.057	.012	.112	.267	70 ± 10	4
Corn oil + .09 g phosphatide	”	.445	.052	.098	.006	.156	.318	66 ± 13	5
Corn oil + .09 g Tween 80	”	.415	.066	.085	.010	.161	.283	64 ± 14	6
Corn oil	Choline deficient	.400	.039	.048	.020	.107	.322	74 ± 4	2

A correction factor of 0.029 g, the mean number of g of total lipid recovered from fasted control rats, has been used in calculating the lipid absorbed.

* Data for corn oil as reported in Reference 1.

TABLE II. Recovery of Oil Preparations in Rats Sacrificed Four Hours After Intestinal Instillation.

Preparation	g lipid admin.	No. of rats	Mean g of lipid recovered	Mean g absorbed	Mean % absorbed ± S.D. of M	Stand. error of mean in %
Corn oil*	.257	8	.233	042	16 ± 5	2
Corn oil + .06 g phosphatide	.287	6	.255	.050	16 ± 6	2
Corn oil + .06 g choline chloride	.257	6	.223	.052	19 ± 4	2
Corn oil + .06 Tween 80	.267	5	.228	.057	20 ± 5	2

A correction factor of 0.018 g, the mean No. of g of total lipid recovered from the intestines of fasted control rats, has been used in calculating the lipid absorbed.

* Data for corn oil as reported in Reference 1.

chloride in water (0.060 g of choline chloride).

Results. Oral feeding experiments. The results of the oral feeding experiments are summarized in Table I. There was no significant change in the absorption of corn oil from the gastro-intestinal tract of the rat after the addition of large amounts of purified soybean phosphatide or Tween 80 (20% of emulsifier for the amount of oil used). Choline-deficient rats absorbed corn oil at the same rate as normal rats. Frozen sections of liver tissue of all of the choline deficient rats taken at the time of sacrifice showed enormous quantities of sudanophilic lipid deposited in the pre-cirrhotic pattern, demonstrating a severe choline deficiency.

Intestinal instillation experiments. The results of the intestinal instillation experiments are summarized in Table II. There was no significant change in the absorption of corn oil in the ligated intestinal segment with the addition of large amounts of purified soybean phosphatide, choline, or Tween 80 (each 20% in respect to the amount of oil used).

Discussion. It is of interest that the purified soybean phosphatide and Tween 80 used in this present work were of no assistance in increasing the absorption of unemulsified fat either after oral feeding or after instillation into the ligated intestine from which gastric, biliary, and pancreatic secretions were excluded, despite the great increase in the intralumenal emulsification potential. The amounts of emulsifiers used were greatly in excess over that needed to make excellent emulsions by mechanical means. Although the intestinal motility of the ligated intestine must certainly have been diminished when compared to the normal, the absorption of 16% of the corn oil instilled into the loop demonstrated that a significant amount of fat absorption had occurred. Since it was found in this laboratory that finely emulsified oil particles of approximately 0.5 μ in diameter can be directly absorbed from the rat intestine(1), it seems apparent that the rats did not make effective use of these exogenous emulsifiers to disperse the ingested oil in their digestive fluids more efficiently than they can do without these emulsifiers, nor did the rats make effective use of these exogenous emulsifiers for oil emulsification when their own biliary and pancreatic juices were excluded from the digestive mixtures in their intestines.

The necessity of choline for the turnover of plasma phospholipids has been adequately demonstrated(12-14) and it has also been reported that choline may represent a limiting factor for the formation of phospholipids in the intestine during the absorption of fat from the intestine(15). However, in the work reported in this paper, fat absorption from the choline deficient rat was normal. Furthermore, an adequate choline supplement did not aid the absorption of fat from the ligated intestine in the normal rat from which most of the digestive secretions which might contain choline were excluded. This suggests that under the conditions used here choline had little function in the absorption of fat from the intestinal lumen and that its participation in the metabolism of ingested fat begins after the absorption of fat from the intestine.

Summary. The absorption of orally fed corn oil in approximately physiological amounts by the rat was unaffected by the presence of amounts of purified soybean phosphatide or Tween 80, greatly in excess over that needed to make excellent emulsions by mechanical means. The absorption of approximately physiological amounts of orally fed corn oil by the severely choline deficient rat was similar to that found in the normal rat. The absorption of corn oil after instillation in the rat intestine ligated below the pancreas and at the ileocecal junction was unaffected by the presence of large amounts of purified soybean phosphatide, Tween 80, or choline chloride.

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Received October 19, 1950. P.S.E.B.M., 1950, v75.

Lack of Direct Biological Conversion of Glycine to Ethanolamine. (18305)

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Stetten(1) obtained N^{15}-labeled ethanolamine upon feeding the corresponding isotopic glycine and suggested that it was formed by the direct reduction of glycine. This hypothesis has found its way into many textbooks and reviews(2). Subsequently Stetten(3) obtained labeled ethanolamine from N^{15}-labeled serine and proposed instead that serine is decarboxylated to yield ethanolamine. The formation of ethanolamine from serine-β-C^{14} has been demonstrated by Levine and Tarver (4) and also by the authors (unpublished experiments). Since glycine can be readily transformed to serine, it appears quite plausible, as has been proposed by Levine and Tarver(4), that ethanolamine is formed from glycine via serine and not directly. However,

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1. Stetten, D., Jr., *J. Biol. Chem.*, 1940, v138, 437; 1941, v140, 143.

2. Jukes, T. H., *In Ann. Rev. Biochem.*, 1947, v16, 193.

3. Stetten, D., Jr., *J. Biol. Chem.*, 1942, v144, 501.

4. Levine, M., and Tarver, H., *J. Biol. Chem.*, 1950, v184, 427.

the reverse is also possible and the isotopic experiments that have been reported do not distinguish between the two possible reaction pathways.

The experiment described below, with C^{14}-carboxyl-labeled glycine was performed to test this point. The results indicate that glycine is not directly reduced to ethanolamine and is so transformed only through serine as an intermediate.

Experimental. Three rats were injected intraperitoneally with 500,000 counts per min. (0.52 mg, 2.8 μc) each of C^{14}-carboxyl-labeled glycine. The animals were killed 4 hours after administration, the livers removed and pooled, and the phospholipids extracted, and then hydrolyzed by refluxing at the boiling point with 3.6% hydrochloric acid for 12 hours. The mixture was chilled, the fatty acids filtered off, and the filtrate concentrated *in vacuo* with repeated addition of water to remove the hydrochloric acid. The residue in about 75 ml of water was treated with neutral aqueous lead acetate, and then freed of excess lead with hydrogen sulfide. The filtrate was evaporated to dryness and extracted with small volumes of ethanol. The alcoholic solution was filtered, evaporated to dryness, and taken up in 1 ml of 1 M hydrochloric acid. The solution was mixed with an excess of calcium oxide and ethanolamine isolated by extraction with ether according to the method of Thierfelder and Schulze* (5). It was counted as the oxalate(6). None of the samples gave counts that were significantly above background.

To the phospholipid extract of the liver of another rat, to which the same dose of carboxyl-labeled glycine had been administered, carrier ethanolamine was added and the base was separated from serine by passage through a column of permutit(7) after acid hydrolysis of the phospholipids. The eluted ethanolamine was treated with periodate and the formaldehyde formed collected as the dimedon derivative. This too contained no activity.

Summary. Ethanolamine isolated following administration of C^{14}-carboxyl-labeled glycine contained no radioactivity. This indicates that glycine is not directly reduced to ethanolamine in the body and that this occurs through the intermediate formation of serine.

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* Choline also was isolated and counted as the chloroplatinate. It contained no radioactivity.

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Received October 21, 1950. P.S.E.B.M., 1950, v75.

A Small Hemagglutinating Component in Preparations of Newcastle Disease Virus.* (18306)

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The size of the virus of Newcastle disease of chicken (NDV) has been estimated to be between 80-120 mμ by filtration through gradocol membranes(1). This general order of size has been confirmed by electron microscopy(2,3), and ultracentrifugation(2,3). The sedimentation studies with virus obtained from infected allantoic fluids indicated that the hemagglutinating activity of NDV is

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* The work described in this paper has been supported by a grant-in-aid from the United States Public Health Service.

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2. Bang, F. B., *J. Exp. Med.*, 1948, v88, 241.

3. Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, v69, 55.

closely associated with the infectious unit(3). However, Burnet noted that upon Seitz filtration of allantoic fluid preparations the infectivity decreased to a far greater extent than the hemagglutinating capacity indicating a "partial dissociation of hemagglutinin and infective activity of NDV"(4).

Studies in this laboratory toward the development of complement fixing antigens for NDV led to observations which appear to substantiate the heterogeneity of NDV hemagglutinins. A comparison of infected allantoic fluids and suspensions of membranes revealed marked differences in the relative sedimentation in the high speed centrifuge of infectivity and hemagglutinins. Whereas in the allantoic fluids both properties sedimented at similar rates, a striking discrepancy became apparent when suspensions of membrane were employed, in that the infectivity was by far more sedimentable than the hemagglutinins. These experiments will be described in detail.

Methods and materials. Virus. The B strain of Newcastle disease virus, originally isolated by Beaudette, was kindly supplied by Dr. Bang. It had been maintained by passage through the allantoic cavity of 10-11-day-old chick embryos. Infected allantoic fluid was kept in glass-sealed ampules in the dry ice chest for stock virus. Suitably diluted seed (10^{-2} to 10^{-6}) was injected allantoically in 0.2 ml amounts. After further incubation of the embryos at 36 to 37°C for 24 to 72 hours the eggs were chilled and the allantoic fluids and membranes harvested aseptically. The allantoic membranes were washed thoroughly in sterile phosphate buffered saline solution of pH 7.2, drained on sterile filter paper, and weighed. A 20% suspension in sterile buffered saline solution was made by emulsifying the tissue for 5 minutes in an ice-cooled Waring blendor. After clarification by preliminary centrifugation of both allantoic fluids and membrane suspensions at 2,000 r.p.m. for 10 to 20 minutes the supernates were centrifuged simultaneously at 20,000 r.p.m. for 20 minutes; the upper two-thirds of the supernatant fluids were saved and the remainder discarded. The sediments were resuspended in buffered saline solution to the original volume. The preparations were found to be stable at 4°C for at least 10 days both as to infectivity and hemagglutinating property.

Infectivity titrations. The virus preparations were diluted in 10-fold steps in sterile brain-heart infusion broth to which 100 units of penicillin and 100 μg of streptomycin had been added per ml. Five 10- to 11-day-old chick embryos each were inoculated allantoically with 0.5 ml of one of the various dilutions. The allantoic fluids from individual eggs were collected separately after an incubation period of 48 hours at 36 to 37°C, the presence of virus was determined by their capacity to cause hemagglutination, and the 50% infectivity end point was calculated according to the method of Reed and Muench. The results are expressed in terms ID_{50}/ml. Since hemagglutinins may under certain conditions escape detection on account of rapid elution of the virus from red cells(5) a number of technics for their demonstration were tried and compared in individual tests. These methods were as follows: (a) to 0.4 ml of undiluted allantoic fluid were added 0.2 ml of a 1% suspension of thrice washed chicken red cells. The mixture was incubated at 4°C for 60 to 90 minutes and the test was read according to the pattern formed by the red cells in the bottom of the test tubes; (b) same as (a) except that the allantoic fluids were diluted 1:10; (c) each allantoic fluid was titrated according to the method described below; (d) one drop each of undiluted allantoic fluid and of a 10 to 20% suspension of chicken red cells were mixed on a glass slide and gently agitated by rotation. In positive samples agglutination became apparent within a few minutes; finally, (e) 0.5 ml of allantoic fluid was mixed with 0.1 ml of M/10 $NaIO_4$ and incubated at room temperature for 30 minutes, when 0.1 ml of a 40% solution of glucose was added in order to neutralize the periodate. After the addition of 0.2 ml of a 1% suspension of chicken red cells the test

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5. Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sc.*, 1942, v20, 81.

was placed at 4°C until the red cells had settled, which on account of the larger total volume, required more time than in methods (a), (b) and (c). All 5 technics gave comparable results provided proper precautions were taken. For methods (a) and (b) the test had to be read as early as possible in order to avoid erroneous interpretation of results on account of elution of the virus; *i.e.*, with increasing delay in reading a "positive" pattern might gradually change to "negative." This did not occur in method (e) which, therefore, is preferred at present because of its independence of careful timing. Method (d), although highly reliable, was somewhat more cumbersome, and method (c) was too elaborate to be practical for the indicated purpose. The results of infectivity titrations presented in the text were determined by at least 2 of the technics and frequently 3.

Hemagglutinin titrations. To serial 2-fold dilutions of the various viral preparations in saline solution, using 0.4 ml volumes, 0.2 ml of a 1% suspension of chicken red cells was added. The test was incubated at 4°C (6) and read in terms of the patterns formed by the cells in the bottom of the tubes. The last initial dilution of the virus preparation giving definite evidence of agglutination was taken as the end point. In most instances the titrations were performed in duplicate and the mean computed. The results are expressed in terms of hemagglutinating (HA) units/ml.

Hemagglutination inhibition tests. Serial 2-fold dilutions of viral preparations were prepared in triplicate, using 0.2 ml volumes. To the first of these series was added 0.2 ml of saline solution; to the second 0.2 ml of 8-fold diluted serum of a patient convalescent from a laboratory infection with NDV(7); and to the third 0.2 ml of 8-fold diluted mumps convalescent serum. After incubation at room temperature for 30 minutes 0.2 ml of a 1% suspension of chicken red cells was added to each tube. The test was incubated at 4°C and read by the pattern formed. Thus, the reduction in hemagglutinin titer by a given dilution of serum was measured. The sera had been treated by $NaIO_4$ according to the method described above in order to minimize non-specific inhibitory activities(8). In addition slide tests were performed by mixing one drop each of undiluted virus preparation, undiluted serum, and 20% suspension of chicken red cells, and the presence or absence of agglutination recorded.

Experimental. Several centrifugation experiments with infected allantoic fluid and membrane preparations were conducted. Since the results were essentially in agreement, only 2 of the protocols will be discussed in greater detail. In the first of the experiments to be described 10-day-old chick embryos were infected with allantoic fluid containing NDV, diluted to 10^{-6}. The allantoic fluids and membranes from half of the living embryos were collected after an incubation period of 48 hours, from the survivors of the other half after 72 hours. In the second experiment a 10^{-2} and a 10^{-6} dilution of the same seed was used and the fluids and membranes were harvested after 48 hours. Emulsification of the tissues and high speed centrifugation were carried out as described in the section on methods and materials. The various preparations in each experiment were centrifuged simultaneously. The results of hemagglutination (HA) and infectivity titrations (ID_{50}) are recorded in Table I.

As can be seen in the table, the infective principle in *allantoic fluid* was largely sedimented by the centrifugal force employed, *i.e.*, only between 1 and 4.5% was left in the supernatant fluid. Sedimentation of hemagglutinins was of a similar order, although the percentage remaining in the supernate was somewhat larger, between 4.6 and 7.7%. This difference may be insignificant in the light of the relative crudeness of the tests employed. The corresponding results with the suspensions of *allantoic membrane*, however, revealed more striking differences. Sedimentation of the infective principle was somewhat greater than in the case of the allantoic fluids, in that only between 0.02 and 0.3% of this

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TABLE I. Differences in Sedimentation of Infectivity and Hemagglutinating Capacity in Allantoic Fluids and Membrane Suspensions.

				Allantoic fluid					Allantoic membrane				
Exp.	Inoculum dilution	Incubation, hr	Material	ID$_{50}$/ml log	% of original	HA units/ml log	% of original	Ratio ID$_{50}$/HA log	ID$_{50}$/ml log	% of original	HA units/ml log	% of original	Ratio ID$_{50}$/HA log
1	10^{-6}	48	Original	10.13		3.51		6.6	9.03		3.46		5.6
			Supernate	8.30	1.5	2.38	7.7	5.9	5.97	.09	2.68	16.6	3.3
			Sediment	>9.80		3.16		6.6	8.33		3.05		5.3
	10^{-6}	72	Original	10.03		3.51		6.5	9.30		3.51		5.8
			Supernate	8.68	4.5	2.30	6.2	6.4	6.80	.3	2.90	25.0	3.9
			Sediment	>9.80		3.28		>6.5	>9.55		3.05		>6.5
2	10^{-2}	48	Original	9.76		3.41		6.4	8.80		3.65		5.2
			Supernate	7.80	1.1	2.20	6.2	5.6	5.68	.08	3.08	26.7	2.6
			Sediment	9.80		3.16		6.6	9.30		3.05		6.3
	10^{-6}	48	Original	9.93		3.41		6.5	8.93		3.51		5.4
			Supernate	7.93	1.0	2.08	4.6	5.9	5.13	.02	2.75	17.5	2.4
			Sediment	9.80		3.11		6.7	9.05		2.98		6.1

activity was retained in the supernate. Removal of the hemagglutinating capacity, on the other hand, was far less pronounced since between 17 and 27% of this property remained suspended. The redispersed sediments derived both from fluids and tissue suspensions indicated fairly complete recovery of the infective principle, whereas a slight loss of hemagglutinin was regularly apparent. Aggregation of particles in the sediment may possibly account for this deficit. These differences in relative sedimentation of the two activities became more evident by a comparison of the ID$_{50}$/HA ratios. Whereas these ratios remained fairly constant for all of the allantoic fluid preparations, *i.e.*, of the order of 10^6, they showed significant variations in the allantoic membrane preparations. The ratios in the resuspended sediments, likewise, were close to 10^6. On the other hand, those found in the supernate were of the order of 10^3 only. The ratios in the original membrane suspensions were somewhat lower than in the corresponding sediments or in the allantoic fluid materials, presumably because of the fact that they contained a mixture of high- and low-ratio materials.†

In further experiments infected allantoic fluids and membrane suspensions were subjected to 3 successive centrifugations at 20,000 r.p.m. for 20 minutes, followed by a fourth run at 30,000 r.p.m. for 1 hour; *i.e.*, the first supernate was used for the second centrifugation, the second for the third, and so forth. The results of ID$_{50}$ and HA titrations in 2 such experiments are shown in Table II. As can be seen, the infectivity in the supernate of allantoic fluid in Experiment 1 had decreased by the third centrifugation to 0.003% and hemagglutination no longer was demonstrable. An increase in the centrifugal force (run No. 4) resulted in little additional sedimentation of infectivity. Again, strikingly different were the results obtained with the suspensions of membranes. After the third and fourth centrifugations only about 0.002% of the original infectivity was left in the supernate, but 5 and 1%, respectively, of the hemagglutinins remained suspended. The ID$_{50}$/HA ratios in the supernates decreased somewhat on successive centrifugation and approached a value of 10^2. Recentrifugation of the resuspended sediments of allantoic fluid or membrane gave similar results in that in the order of 95% of the hemagglutinins were found sedimentable in both instances (see Table IV).

These observations reveal a definite discrepancy between sedimentation of infectivity and hemagglutinins from allantoic fluid, on the one hand, and from allantoic membrane

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† Similar experiments were conducted recently with the Victoria and California No. 11914 strains of NDV with essentially similar results.

TABLE II. Effect of Repeated Centrifugation Upon Infectivity and Hemagglutinating Capacity of Allantoic Fluid and Allantoic Membrane Suspension.

				Allantoic fluid					Allantoic membrane				
Exp.	Inoculum dilution	Incubation, hr	Material	ID$_{50}$/ml log	% of original	HA units/ml log	% of original	Ratio ID$_{50}$/HA log	ID$_{50}$/ml log	% of original	HA units/ml log	% of original	Ratio ID$_{50}$/HA log
1	10^{-6}	48	Original	9.98		3.58		6.4	9.08		3.71		5.4
			supernate										
			1st	7.93	.9	2.20	4.2	5.7	5.68	.04	2.98	18.7	2.7
			2nd	6.30	.02	.70	.1	5.6	4.69	.004	2.68	9.3	2.0
			3rd	5.47	.003	<.07	<.1	—	4.30	.002	2.38	4.7	1.9
			4th	5.07	.001	<.70	<.1	—	4.30	.002	1.60	0.8	2.7
2	10^{-6}	48	Original						8.47		3.41		5.1
			supernate										
			1st						4.93	.03	2.81	25.0	2.1
			2nd						4.56	.01	2.51	12.5	2.1
			3rd						3.68	.002	2.38	9.3	1.3

suspensions, on the other hand. The results seem to indicate that in the membranes, and possibly to a lesser extent also in allantoic fluids, 2 distinct hemagglutinating components may be found as a result of infection with NDV. One of these appears to be inseparable from, and therefore probably indentical with the infectious principle, the other seems to be of smaller size and probably noninfectious. This explanation has not been contradicted by a number of subsequent experiments which were devised to substantiate further this suggestion.

That both particles are products of NDV infection could be shown by hemagglutination inhibition tests. As can be seen in Table III the hemagglutinating capacity of both allantoic fluid and membrane preparations, as well as of the corresponding supernatant fluids, obtained after high speed centrifugation, were largely neutralized by a serum obtained from a patient convalescent from an infection with NDV whereas mumps convalescent serum failed to do so. In the slide test the specific serum completely prevented visible agglutination of the red cells by all virus materials tested. In the tube test the HA titers were reduced to a fraction of 1% in the cases of the more potent virus preparations and the tests became negative in the supernates showing lesser titers of hemagglutinins. These results clearly indicate a relationship between the non-sedimentable hemagglutinins and NDV.

TABLE III. Specificity of Hemagglutinins in Allantoic Fluid and Membrane Preparations.

Material	Serum	Slide test	Tube test HA units/ml
Allantoic fluid			
Original	Saline	+	1920
	Anti NDV	—	5
	" mumps	+	1920
Supernate 20,000 r.p.m.	Saline	+	80
	Anti NDV	—	<2.5
	" mumps	+	40
Allantoic membrane			
Original	Saline	+	960
	Anti NDV	—	2.5
	" mumps	+	960
Supernate 20,000 r.p.m.	Saline	+	120
	Anti-NDV	—	<2.5
	" mumps	+	120

It had been shown in the past that the medium in which the virus is contained may influence its shape; that is, when suspended in allantoic fluid or distilled water it assumes a spherical form, whereas in buffered saline solution it appears filamentous(9). In the light of this information attempts were made to interchange, as far as possible, the media of the two virus preparations prior to centrifugation studies. Consequently, in one set of experiments equal parts of normal allantoic fluid were mixed with suspensions of infected membranes, and, conversely, suspen-

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9. Bang, F. B., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 135.

TABLE IV. Effect of Medium on Sedimentation of Hemagglutinating Capacity in Allantoic Fluid and Allantoic Membrane Suspension.

		Allantoic fluid				Allantoic membrane			
		HA units/ml				HA units/ml			
Material	Medium added	Original	Supernate	Sediment	% of original in supernate	Original	Supernate	Sediment	% of original in supernate
Original	0	7680	240	1920	3.1	5120	960	1920	18.7
”	Saline sol.	1920	80	960	4.2	2560	320	480	12.5
”	Norm. mem. susp.	1920	80	1920	4.2	1920	320	960	16.6
”	Norm. fluid	1920	80	960	4.2	1920	480	960	25.0
Sediment 20,000 rpm	Buffered saline	2560	120	1920	4.7	—	—	—	
” ”	Norm. mem. susp.	3840	120	3840	3.1	—	—	—	
” ”	Buffered saline	2560	80	1920	3.1	1920	60	1280	3.1
” ”	Distilled H_2O	3840	60	3840	1.5	1920	120	1920	6.2

sions of normal allantoic membranes were added to infected allantoic fluids. In a second series the sediments obtained by high speed centrifugation from aliquots of an infected allantoic fluid preparation were redispersed in a suspension of normal allantoic membrane, in distilled water, or in buffered saline solution. Similarly, sediments from infected membranes were suspended in distilled water or buffered saline solution. These various preparations were then subjected to centrifugation at 20,000 r.p.m. for 20 minutes and the various fractions tested for hemagglutinating activity. When the corresponding preparations were compared no significant differences became apparent in the sedimentation of hemagglutinins regardless of the medium in which they were suspended. These results are tabulated in Table IV. As far as has been ascertained, changes in the medium were without apparent influence upon sedimentation.

Furthermore, attempts were made to determine whether non-specific inhibitors of hemagglutination might have played a role in the experiments. Treatment of infected allantoic fluids and membrane suspensions with receptor destroying enzyme (RDE) as contained in culture filtrates of *V. cholerae*(10), or with $NaIO_4$(8), failed to change significantly the results of hemagglutination tests as compared to those obtained with untreated preparations. It was noted, however, that the hemagglutinin titers in membrane preparations after periodate treatment were on the whole lower but the percentage of hemagglutinins remaining in the supernate after high speed centrifugation was similar to that left suspended in untreated preparations.

Finally efforts were made to separate, if possible, the two types of hemagglutinating components by Seitz filtration. Infected allantoic fluids, as well as supernates and sediments of membrane suspensions obtained by high speed centrifugation were passed through Seitz E. K. filters of the Boerner type in 2 successive portions of 10 ml each. Whereas the allantoic fluids passed without great delay, difficulties were encountered with the membrane preparations and prolonged centrifugation was required to filter the amounts needed. Hemagglutination tests with these materials, as summarized in Table V, Section A, showed that no hemagglutination became measurable in the filtrates of the allantoic fluids and the high speed sediments of the membranes under the conditions of the experiments. On the other hand, filtration of the high speed supernates of the membrane suspensions clearly resulted in passage of hemagglutinins. These data would seem to be in agreement with the suggestion that two hemagglutinating units are formed in the in-

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10. Burnet, F. M., McCrea, J. F., Stone, J. D., *Brit. J. Exp. Path.*, 1946, v27, 228.

TABLE V. Seitz Filtration of Allantoic Fluid and Membrane Suspensions.

Material	HA units/ml in			ID_{50}/ml in	
	Original	1st filtrate	2nd filtrate	Original log	1st filtrate log
A. Allantoic fluid (1) Original	1280	<5	<5		
,, ,, (2) ,,	5120	<5	<5		
,, ,, (3) ,,	2560	<5	<5		
Allantoic membrane (1) Supernate 20,000 rpm	480	120	320		
,, ,, Sediment in buffered saline	320	<5	<5		
,, ,, (2) Supernate	320	160	320		
Sediment in buffered saline	960	<5	<5		
B. Allantoic fluid (4) Original	2560	<5	<5		
20,000 rpm					
,, ,, Supernate	320	<5	<5		
,, ,, Sediment in buffered saline	1280	<5	<5		
,, ,, Sediment in NMS	1280	320	640		
Allantoic membrane (4) Original	7680	240	480		
20,000 rpm					
,, ,, Supernate	1280	960	1920	6.04	5.63
,, ,, Sediment in buffered saline	2560	<5	<5	8.80	<2.30
,, ,, Sediment in NMS	3840	120	320	8.68	6.82
C. Allantoic fluid (4) + Saline Solution Original	3840	<10	<10	9.13	2.52
,, ,, + NMS Original	3840	80	960	9.52	7.97

fected chick embryo, one readily filtrable and found mainly in the membranes, the other largely retained by the Seitz filter and contained in the allantoic membranes as well as the fluids. However, since filtration is readily affected by various physical factors it was thought advisable to study the influence on Seitz filtration of the addition of supernatant fluid obtained from normal allantoic membranes by high speed centrifugation (NMS) to non-filtrable preparations of hemagglutinins. The method of filtration in these experiments was similar to that described in the previous experiments with the exception that smaller filters with a capacity of 2 ml were employed. As shown in Table V, section B, resuspension in NMS of hemagglutinins sedimented from allantoic fluids or emulsified membranes permitted their passage through the filter, whereas, when suspended in buffered saline solution, they failed to come through. Similarly, when NMS was added in equal volumes to infected allantoic fluids, hemagglutinins filtered through the Seitz pad quite readily (Table V, Section C). Thus, these experiments were unrevealing as far as differentiation between hemagglutinins was concerned. The infectivity titrations, where done, showed that the infective principle as well as the hemagglutinins passed through the filter in the presence of NMS.

Discussion. The hemagglutinins of various viruses have been found to be closely associated with the respective infectious principles, except for those observed in the pox group of agents(11). However, recently some evidence has been presented which indicates that early in the infectious cycle of influenza virus in the chick embryo a hemagglutinin appears which seems to be non-infectious as yet and which is considered an "immature" or "developmental stage" in virus production (12). Furthermore, injections of influenza virus into tissues, which, as a rule, do not support propagation of the agent to the extent of liberation of fully infectious virus, nevertheless may lead to the development of "incomplete" forms, *i.e.*, a non-infectious, hemagglutinating component(13). Upon

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11. Burnet, F. M., and Stone, J., *Austral. J. Exp. Biol. and Med. Sc.*, 1946, v24, 1

12. Henle, W., and Henle, G., *J. Exp. Med.*, 1949, v90, 23.

analytical centrifugation of influenza virus preparations, obtained under various conditions, 2 components were noted, both resulting from the infection, one identical with the infectious principle, the other smaller but apparently endowed with hemagglutinating activity(14,15). This material has been considered a "precursor" of fully active virus (15). Neither the "immature" or "incomplete" forms nor the "precursor" have been isolated as yet for more extensive analysis of their physical and biological properties.

The data presented above, which confirm and extend earlier observations by Burnet(4), may be considered in the light of the problems raised by the studies on influenza virus. The available information appears to indicate the presence in chick embryos infected with NDV of 2 hemagglutinating components, one associated with the infectious principle, the other distinctly smaller and according to all indications non-infectious. As in the influenza problem, the question arises whether this smaller component represents a breakdown product of the seed virus or of the new generations of the agent, or whether they constitute a developmental stage in virus reproduction. The fact that this component is found mainly in the infected tissue and not in the allantoic fluid into which the new generations are liberated, seems to favor the latter hypothesis.

Summary. Disproportionate sedimentation in the high speed centrifuge of hemagglutinins and infective principle of Newcastle disease virus in suspensions of infected allantoic membranes has been reported. This suggests the presence in such preparations of two hemagglutinating particles of different sizes; one of these appears to be identical with the infectious principle, the other, smaller one, according to all indications is non-infectious. The specificity of this smaller hemagglutinin has been established by hemagglutination-inhibition tests. The medium in which the virus is suspended apparently does not effect the sedimentation of hemagglutinating capacity. Experiments employing Seitz filtration have been unrevealing.

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14. Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, v79, 301.

15. Gard, S., and von Magnus, P., *Ark. Kemi, Miner. och Geol.*, 1947, v24b, No. 8.

Received October 23, 1950. P.S.E.B.M., 1950, v75.

Studies of Newcastle Disease Virus (NDV) Propagated in the Cave Bat (*Myotus lucifugus*). (18307)

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The egg-adapted Newcastle disease virus (NDV) strains: California, Colorado, Delaware, Kentucky, Minnesota, and Connecticut, used for these studies were obtained in 1946 from the Bureau of Animal Industry, Department of Agriculture, Washington, D. C. These strains were isolated by the Bureau from infected chicken tissue from the corresponding states before the introduction of commercial live virus vaccines for Newcastle disease.

The cultures had been carried in 10-day embryonated chicken eggs through 6 serial passages. In our laboratory they were carried 6 additional passages in 10-day embryonated White Leghorn eggs. Embryo passage of the California strain was continued through the 24th passage. Allanto-amniotic fluid from several eggs dead of each strain was harvested and pooled separately for each strain. All strains of the virus were confirmed to be NDV by neutralization tests conducted in 10-day embryonated chicken eggs. The tests were performed according to the procedure recommended by the Bureau of Animal Industry(1). Newcastle-immune chicken serum neutralized the virus in all cases while normal chicken serum had no effect. Five-hundredths ml of pooled, infected allanto-amniotic fluid from embryonated egg passages of all strains was inoculated in the right frontal lobe of each of 4 cave bats. Within 4 to 6 days the bats from all strains showed typical Newcastle virus symptoms: irritability, malaise, rhythmical contraction of muscles starting in the limbs and spreading over the entire body, paralysis of the hind legs, prostration, and death. There was no evidence of excessive salivation from pharyngeal paralysis which is characteristic in other animals infected with the hamster-adapted NDV California strain 11,914(2). When nervous symptoms developed in each bat, the brain was removed aseptically, ground with alundum, and diluted to a 10% suspension with physiological saline. The brain suspension from each strain was inoculated into the allantoic sac of 10-day embryonated eggs. All embryos died within 72 hours. Pooled allanto-amniotic fluids from dead eggs of each strain was prepared. Neutralization tests were done on these infected pools against NDV antiserum and normal chicken serum using embryonated chicken eggs as the test host. The results of these tests proved the virus in all strains to be that of Newcastle disease.

The California strain of egg-adapted NDV was carried through 10 passages in the cave bat as shown in Table I. Brain material for passage was chosen from bats sacrificed when nervous symptoms developed. Amounts of 0.05 ml of 10% brain suspension from the preceding passage were injected intracerebrally throughout the 10 passages. These passages are being continued at the present time. Brain material from the 2nd intracerebral bat passage was injected into 10-day embryonated eggs, and upon death of all embryos in 72 hours the allanto-amniotic fluid was harvested, concentrated, and examined under the Emu electron microscope. The virus particles showed morphological characteristics similar to those previously described by the authors(3).

TABLE I. Bat Passage of a Strain of Egg-Adapted Newcastle Disease Virus (California Strain No. 11,914).

Passage No.	No. bats inoculated	No. bats paralyzed, moribund, or dead	Min. to max. incubation period (days)
1	4	3	4-6
2	6	6	4-6
3	8	5	3-5
4	4	3	2-4
5	6	6	2-5
6	8	6	3-4
7	6	4	3-4
8	6	4	2-4
9	6	6	2
10	10	10	3-4

TABLE II. Results of Neutralization Test of Bat-Adapted NDV (10th Passage).

Material inoculated	Virus dilutions 10^{-1}	10^{-2}	10^{-3}
Virus bearing brain material	4/4	2/4	2/4
Same + normal chicken serum	3/4	3/4	2/4
Same + Newcastle-immune chicken serum	0/4	0/4	0/4

Numerator of fraction denotes the number of deaths.

Denominator of fraction denotes the number of bats inj.

A virus neutralization test was conducted using brain suspension of the 10th intracerebral bat passage with specific Newcastle-immune and normal chicken sera and with bats as the test host. Specific immune sera completely neutralized the virus while normal chicken sera did not. The virus titered $10^{-2.5}$ according to the Reed-Muench method of calculation(4). Details are given in Table II.

Summary. The cave bat, *Myotus lucifugus*, is susceptible by intracerebral inoculation to the 6-egg-adapted NDV strains: California, Colorado, Connecticut, Delaware, Kentucky, and Minnesota, used in this study. The California strain was carried 10 passages in the bat by intracerebral inoculation. Serial passage in bats was not attempted with any of the other 5 strains.

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3. Reagan, R. L., Smith, E. J., and Brueckner, A. L., *J. Bact.*, 1950, To be published.

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Received October 23, 1950. P.S.E.B.M., 1950, v75.

Characterization of a Fluorescent Lipid Fraction From Cancer-bearing Animals.[*] (18308)

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In 1947 Penn and Kaplan(1) confirmed Penn's previous isolation of a "methylcholanthrene-like" substance from cancerous human livers(2). Because of the great interest attached to the isolation of endogenous carcinogens, other authors have extended Penn's investigations. Jones and Jamieson (3) prepared fractions by methods "which parallel quite closely those employed by Penn", but were unable to detect in them the typical ultraviolet spectra of polynuclear aromatic hydrocarbons. They imputed the fluorescence observed by Penn to iso-anhydrovitamin A which they detected in some of their preparations. Tuttle and Jacobs(4) were unable to find the typical ultraviolet absorption of methylcholanthrene in crude fractions supplied by Penn. Since far smaller amounts of material can be detected by fluorescence spectrophotometry than by ultraviolet absorption this work does not preclude the presence of a very small amount of a fluorescent "methylcholanthrene-like" substance in Penn's extracts. The present study was undertaken to investigate further the fluorescent components of liver lipids extracted by Penn's procedure.

Materials and methods. Two human livers were obtained through the kindness of Dr. E. Stern. The tissue was digested, extracted and saponified according to the directions of Penn, except that a second hydrolysis of the unsaponifiable fraction was omitted after it was demonstrated that this procedure did not remove any further material. The crude unsaponifiable fraction was further purified by treatment with phthalic anhydride and Girard's reagent T according to the directions of Dobriner, Lieberman and Rhoads(5). This procedure was shown not to affect the blue fluorescence of the material under investigation, and it offered the advantage of eliminating more contaminating compounds than the digitonin treatment employed by Penn. The small amount of tan oil remaining was further fractionated by chromatography on alumina (Alcoa F-20, washed with 5% acetic acid and reactivated at 240°) and silicic acid (Merck, screened to 140 mesh and activated at 350°) from petroleum ether (30-60°, redistilled from sulfuric acid) solution.

Rabbit tissues. Rabbit livers and blood, from animals implanted with the Brown-Pearce carcinoma, were obtained through the courtesy of Dr. E. M. Jacobsen. Only uninvolved tissue was used. The livers were extracted exactly as was the human material. Rabbit blood was poured into 4 volumes of a 1:3 ether-ethanol mixture. The suspension was filtered and the precipitate re-extracted with fresh ether-ethanol mixture. The solvents were removed on the steam bath and the residue extracted into ether. The residue after evaporation of the ether was treated further like the liver lipids. Unsaponifiable lipids, freed of hydroxylic and ketonic compounds, were chromatographed on alumina from petroleum ether. Blue-fluorescent material was eluted with 3-10% ether in petroleum ether until contamination with brown-fluorescent material became evident. The eluates were then re-chromatographed from petroleum ether on silicic acid and the column washed thoroughly with petroleum ether. Elution with 0.5% ether in petroleum ether brought down the blue-fluorescent zone which could not be further purified by chromatography.

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[*] This study was supported by a grant from the Cancer Research Coordinating Committee of the University of California. The authors wish to express their gratitude to Dr. T. A. Geissman for his aid and interest in the work, and to Dr. M. Kasha for help in the interpretation of the fluorescent spectra.

[†] Deceased August 28, 1949.

1. Penn, H. S., and Kaplan, J., *Nature,* 1947, v160, 18.

2. Penn, H. S., *Nature,* 1942, v149, 193.

3. Jones, R. N., and Jamieson, J. R., *Nature,* 1947, v160, 677.

4. Tuttle, W. P., and Jacobs, T. L., unpublished data.

5. Dobriner, K., Lieberman, S., and Rhoads, C. P., *J. Biol. Chem.,* 1948, v172, 248.

Results. Fluorescent material was isolated from human liver, rabbit livers and blood (both from normal and from cancer-bearing animals) and normal hog blood. In "mixed chromatograms" on alumina and on silicic acid the human liver material formed single sharp zones with hog and rabbit blood fractions. A systematic attempt to compare the properties of the blue-fluorescent lipid fractions with authentic methylcholanthrene was undertaken:

1. When methylcholanthrene was mixed with the fluorescent material from "cancerous" rabbit blood and the mixture chromatographed on silicic acid, the two constituents separated. The methylcholanthrene was more strongly adsorbed on the column and the lipid could be washed out selectively. The eluate showed the fluorescence spectrum of the lipid material only.

2. Since Dimter(6) and Stanger, Steiner and Bolyard(7) had isolated hepene (a highly unsaturated terpene precipitable by the addition of ethereal hydrogen chloride) from human livers, the effect of this reagent on methylcholanthrene and our fluorescent liver fraction was investigated. No precipitate formed in either case. The blue fluorescence of the rabbit lipid was quenched instantaneously and could be completely restored by neutralization of the solution with base. Under the same conditions the fluorescence of authentic methylcholanthrene persisted for about a minute and then was gradually replaced by a canary-yellow fluorescence which surpassed the original blue fluorescence in intensity. Neutralization resulted in a blue fluorescence of much lower intensity than the original sample showed.

3. Jones and May(8) attempted unsuccessfully to concentrate the active fluorescent constituent of human livers by precipitation as the picric acid complex. We, too, failed to obtain any adsorption on the precipitate with our lipid fractions. But authentic methylcholanthrene also could not be concentrated from solutions showing equal fluorescence in which the concentration of the carcinogen was about 10^{-8} M.

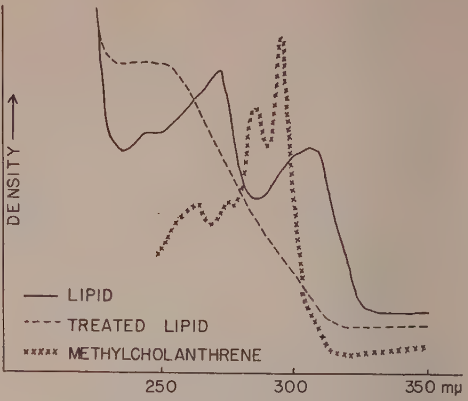

FIG. 1. Ultraviolet Spectra.

All spectra were determined on a Beckman DU spectrophotometer. The ordinate represents optical density plotted in arbitrary units so as to obtain comparable absorptive intensity for the three substances represented. Lipid refers to the unsaponifiable material which was eluted from a silicic acid column as a single sharp zone. Treated Lipid refers to the same material after treatment with potassium permanganate in acetone solution. Methylcholanthrene was obtained from the Eastman Kodak Company. It was purified by chromatography on silicic acid and was then treated with permanganate in acetone under the same conditions as described for the lipid.

4. The ultraviolet absorption spectrum of the fluorescent lipid was determined. Pooled samples from human and rabbit sources gave rise to peaks at 272 mμ and 306 mμ. (Fig. 1.) The curve did not resemble the spectrum of methylcholanthrene at all. Treatment of the lipid fraction with potassium permanganate in acetone did not affect the blue fluorescence of the lipid, but it completely eliminated the absorption peaks in the ultraviolet spectrum. Similar treatment of a sample of authentic methylcholanthrene left the absorption of this hydrocarbon completely unchanged.

5. Since Jones and Jamieson(3) had sug-

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6. Dimter, A., *Z. physiol. Chem.*, 1941, v271, 293.

7. Stanger, D. W., Steiner, P. E., and Bolyard, M. N., *J. Am. Chem. Soc.*, 1944, v66, 1621.

8. Jones, R. N., and May, C. D., *Cancer Res.*, 1944, v4, 304.

gested that the banded fluorescence of Penn's liver extracts might arise from a carotenoid compound, a sample of pure dehydro-vitamin A, obtained through the kindness of Dr. E. Schantz of Distillation Products, Inc., was investigated. It differed from the lipid material and from methylcholanthrene both in showing only a weak yellow fluorescence and in its great instability toward atmospheric oxygen, toward which the tissue extracts and methylcholanthrene were entirely stable.

6. Penn first investigated the fluorescence of lipid fractions from cancerous and normal humans. He found marked banding in the spectra of the "cancerous" lipids and only diffuse fluorescence in normal lipids. Visually the two types could not be differentiated. Krichesky and Jacobsen(9) observed similar banding in the fluorescent spectra of unsaponifiable fractions of rabbit blood. In the present study only general blue fluorescence could be obtained from human liver and hog blood lipids. One of the four pools of blood from Brown-Pearce carriers also showed no banding. The other three pools of "cancerous" blood and the one sample of normal rabbit blood investigated gave rise to three distinct bands to the long wave length side of the 365 mμ mercury line. The intensity of the fluorescence from normal rabbit blood seemed to be somewhat lower than that from the blood of Brown-Pearce carriers. Methylcholanthrene gave bands which were very similar in general appearance to those obtained from the banded lipids, but the bands observed with the lipid fractions were shifted 34 mμ toward the ultraviolet.

Discussion. The lipid fractions here investigated obviously are still mixtures of several chemical compounds. Penn and Kaplan reported that most of their material did not absorb hydrogen on catalytic microhydrogenation, and thus could not give rise to the fluorescence observed. Penn(10) found high vacuum fractionation useful for purification of the lipid fraction, but it did not remove the saturated companion substances.

The inhomogeneity of the fractions makes it difficult to evaluate the significance of our findings. However, the separation of added methylcholanthrene from rabbit blood lipid on a silicic acid column and the marked differences in behavior toward ethereal hydrogen chloride preclude identity of the substances. The total absence of the typical methylcholanthrene ultraviolet spectrum in the most highly purified sample of lipid sets an upper limit to the concentration of the methylcholanthrene which could conceivably be present. The banded fluorescent spectrum of the lipid fractions differs significantly from that of authentic methylcholanthrene. The shift of 34 mμ precludes identity of the two substances. Finally it is interesting to note that the compound giving rise to the banding in the fluorescent spectrum is not invariably a constituent of the tissues of cancer-bearing animals. It also occurs in analogous fractions from normal animals. In this respect it resembles the equally sporadic carcinogenic factor isolated from human livers by Steiner (11).

Conclusion. While the blue-fluorescing material first isolated by Penn from human livers has not been identified, several differences between it and methylcholanthrene have been demonstrated. The material occurs in the tissues of both normal and cancer-bearing animals, and its relationship to carcinogenesis is doubtful.

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9. Krichesky, B., and Jacobsen, E. M., unpublished data.

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Received October 23, 1950. P.S.E.B.M., 1950, v75.

Histochemical Studies of Phosphatases in the Nervous System of Thiamine Deficient Pigeons. (18309)

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(Introduced by E. W. Dempsey.)

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Thiamine deficiency is known to produce definite pathological alterations in the nervous system: capillary changes predominantly around the ventricular system(1), degeneration of the primary neurons of the proprioceptive nervous system and, finally, of the efferent nervous system(2) and corpus striatum(3). Since the changes in phosphatases in the nervous tissue in thiamine deficiency have not hitherto been investigated, it seemed desirable to do so.

Methods. The group of 14 pigeons used in this experiment was divided as follows: 3 served as normal controls, while 11 were fed on a thiamine deficient diet, consisting of cleaned rice thoroughly washed, to which 0.2 cc cod liver oil was added every 2 days. Some of the pigeons were killed after 14 days, but others were given the deficient diet for 25 to 29 days until typical opisthotonus appeared. Several of the latter were given daily injections of thiamine (15 to 45 gamma) for 3 days and 7 days. All of the pigeons were decapitated. Spinal ganglia and blocks of tissue obtained from the medulla oblongata, cerebellum and spinal cord were fixed in chilled acetone. Both alkaline and acid phosphatases were demonstrated histochemically by the Gomori-Takamatsu method as modified slightly by Shimizu and Arizono(4). Sodium glycerophosphate was used as substrate. The sections were incubated at pH 9.2 for 3 hours for alkaline phosphatase, and at pH 4.8 for 6 hours for acid phosphatase.

Results. Alkaline phosphatase. In the central nervous system of normal healthy pigeons alkaline phosphatase is distributed in a pattern similar to that seen in mammals, except that only traces of the enzyme occur in the walls of the blood vessels. In general, the grey matter presents a stronger reaction than the white matter. The neuropil of the grey substance reacts much more pronouncedly than the nerve cells (Fig. 1). Moreover, the enzyme content of the cerebral substance near the surface of the brain and cerebral ventricles is greater than that of the internal structures, as already reported by Shimizu(5).

Thiamine deficiency in pigeons causes characteristic changes in the distribution of alkaline phosphatase, which are most evident in the ventral horns of the spinal cord (Fig. 2). There is an apparent increase in the amount of enzyme in the ventral horn cells, accompanied by moderate shrinkage of the cell bodies and no change, or only a slight increase, in the reaction of the neuropil. As a consequence, the contrast between nerve cells and neuropil observed in the normal brain disappears. Following injection of thiamine, however, the reaction of the nerve cells decreases, whereas the reaction of the neuropil remains unchanged (Fig. 3), resulting in a nearly complete restoration of the normal condition.

In normal pigeons the grey matter in the floor of the fourth ventricle and outer surface of the brain reacts intensely, while the internal structures, as, for example, the formatio reticularis, exhibit only slight activity. In thiamine deficiency of maximal duration there is a general increase in enzymatic activity involving both neuropil and nerve cells, a change which is especially evident in the formatio reticularis. Following administration of thiamine, restoration to normal takes place in the same manner as in the spinal cord.

The cerebellum of thiamine deficient pigeons

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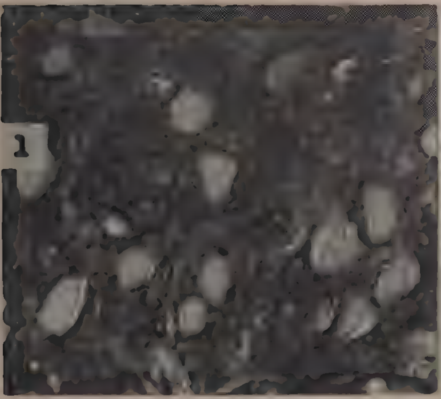

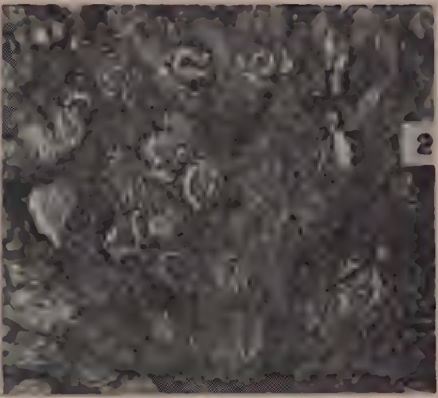

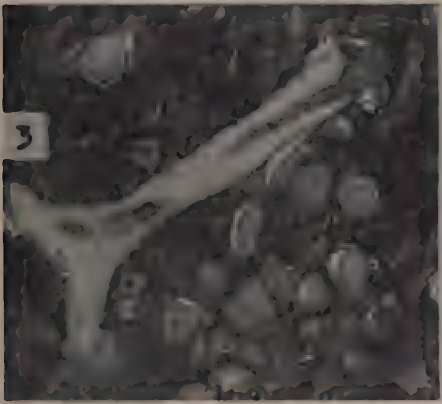

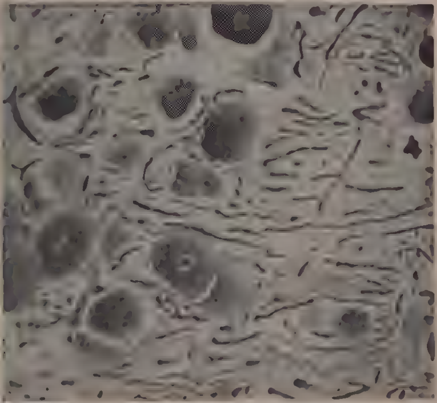

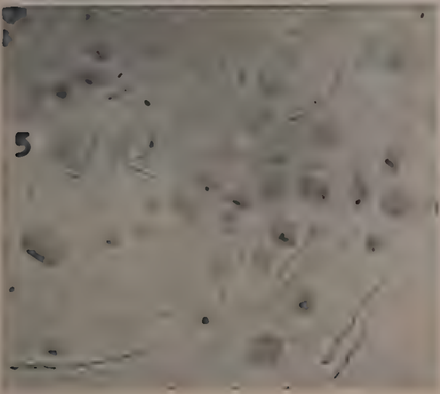

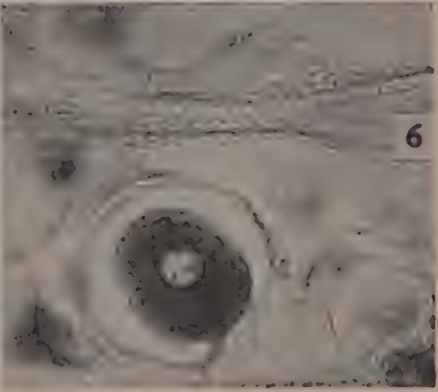

All of the figures illustrate the histochemical distribution of phosphatases in the nervous tissues of the pigeon. The blocks of tissue were fixed in chilled acetone. The sections were incubated in sodium glycerophosphate at pH 9.2 for 3 hours for alkaline phosphatase, and at pH 4.8 for 6 hours for acid phosphatase.

FIG. 1. Alkaline phosphatase reaction in the ventral horn of the spinal cord of a normal pigeon. Observe the marked reaction of the neuropil and the faint reaction of the nerve cells. × 130.

FIG. 2. Alkaline phosphatase reaction in the ventral horn of a pigeon fed on a thiamine

deficient diet for 29 days. Compare with Fig. 1. The shrunken ventral horn cells show a markedly increased phosphatase reaction, whereas the intensity of staining of the neuropil has not changed. × 130.

FIG. 3. Alkaline phosphatase reaction of ventral horn of a pigeon fed on a thiamine deficient diet for 29 days followed by daily injections of thiamine for 7 days. The reaction in the nerve cells apparently has diminished, so that the cells have been nearly restored to normal, while the neuropil exhibits a slightly increased response. × 130.

FIG. 4. Acid phosphatase reaction of a spinal ganglion of a normal pigeon. The nerve cells and axis cylinders show moderate activity. The pattern of the neurokeratin in the myelin sheaths is only faintly visible in few places. × 200.

FIG. 5. Acid phosphatase reaction of spinal ganglion of pigeon fed on thiamine deficient diet for 29 days. A marked decline in phosphatase has occurred in both nerve cells and axis cylinders. × 200.

FIG. 6. Acid phosphatase reaction of spinal ganglion following daily injection of thiamine for 7 days to thiamine deficient pigeon. The nerve cells and axis cylinders have reacquired acid phosphatase. Note the appearance of a neurokeratin-like network around the axis cylinders. × 500.

also presents a general increase in activity involving a pronounced reaction of the shrunken Purkinje cells.

In the spinal ganglia the ganglion cells exhibit a moderate increase in the amount of enzyme, while the capsular cells, in most instances, show a decrease.

Acid phosphatase. Moderate amounts of acid phosphatase are demonstrable uniformly in both axis cylinders and nerve cells in healthy pigeons (Fig. 4). Although the neurofibrils of nerve fibers contain acid phosphatase, as demonstrated by Wolf, Kabat and Newman(6), the neurofibrils of the nerve cell bodies are not always positive; instead, nerve cells may contain coarser networks resembling a Golgi apparatus. A network resembling the pattern of neurokeratin is occasionally barely perceptible in the myelin sheaths surrounding some of the nerve fibers.

In thiamine deficient pigeons a decrease in acid phosphatase takes place, more apparent in the axis cylinders than in the nerve cells (Fig. 5). This characteristic change is evident in all regions examined. Following administration of thiamine, the enzymatic activity of the nerve fibers is restored to a moderate degree, while that of the nerve cells is variably restored, being slight in some cells and very pronounced in others.

After injection of thiamine, it is observed that the network in the myelin sheaths, resembling that of neurokeratin, sometimes becomes more distinct than normal, as, for example, in the nerve fibers of the spinal cord (Fig. 6).

The Purkinje cells of the cerebellum normally show a moderate cytoplasmic reaction and a faint nuclear one. However, in thiamine deficiency the reaction of the cytoplasm is diminished, whereas that of the nuclei is increased. These changes are also observed, although less markedly so, in other types of neurones.

Comment. In the mammalian central nervous system alkaline phosphatase occurs mainly in the walls of the blood vessels at the site of the blood brain barrier(7). In pigeons there is much less present in the blood vessel walls, the major reaction being in the neuropil. The reaction in the nerve cells of normal pigeons is relatively slight, but in thiamine deficiency the amount of enzyme in these cells becomes greatly increased, so that the contrast with the surrounding neuropil diminishes.

Acid phosphatase of nerve tissue is apparently reduced by thiamine deficiency, a result related possibly to the observation of Hard and Lassek(8) that this enzyme disappears from axis cylinders after removal of their cells of origin. Following thiamine administration to thiamine deficient pigeons, the acid phosphatase reaction sometimes brings out a network in the myelin sheaths resembling the pattern of neurokeratin. This is of interest since acid phosphatase is assumed by some(8) to participate in the

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6. Wolf, A., Kabat, E., and Newman, W., *Am. J. Path.*, 1943, v19, 423.

7. Wislocki, G. B., and Dempsey, E. W., *J. Comp. Neur.*, 1948, v88, 319.

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maintenance of the myelin sheaths.

Summary. Nervous tissues of thiamine deficient and control pigeons were examined histochemically in respect to both alkaline and acid phosphatases. In this deficiency the nerve cells shrink and acquire increased amounts of alkaline phosphatase, whereas the neuropil shows only a slightly increased reaction. Acid phosphatase of the axis cylinders and cytoplasm of the nerve cells is diminished in amount. After administration of thiamine to the deficient pigeons, a considerable return to normal is observed. Accompanying restoration of the acid phosphatase a network, similar to the pattern of "neurokeratin", becomes evident in the myelin sheaths.

Received October 25, 1950. P.S.E.B.M., 1950, v75.

Failure to Demonstrate Antibody in Feces of Monkeys Vaccinated with Poliomyelitis Virus, Lansing Strain.[*][†] (18310)

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There is evidence that antibodies for various infectious agents occur in certain of the secretions and excretions of humans and animals. For example, antibodies have been found for influenza virus(1-3) and the Lansing strain of poliomyelitis virus(4-6) in nasal secretions. In feces, antibodies have been reported for cholera(7,8), several enteric bacilli(9-11) and possibly for one strain of the Lansing type of poliomyelitis virus(12).

The present study was made in order to determine whether antibodies occurred in the feces of monkeys during and after vaccination with the Lansing strain of poliomyelitis virus. Monkeys were chosen for this study since it has been shown that high antibody titers may be obtained in their sera by vaccination(13,14).

Materials and Methods. Eight young Macaca mulatta monkeys were used. The monkeys were examined daily, and daily rectal temperatures were recorded. For titration of the virus-neutralizing capacity of stool suspensions and serum specimens, white Swiss mice 3-4 weeks of age and 12-16 g in weight were used; these were examined daily for a month. The day of onset of paralysis, the character of the paralysis and the day of death were recorded. At the end of one month all survivors were discarded.

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[*] Presented to the Soc. Am. Bact., Baltimore, Md., May 14, 1950.

[†] Aided by a grant from the National Foundation for Infantile Paralysis.

[‡] Fellow in the Medical Sciences, National Research Council, 1947-48.

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12. Hammon, W. McD., *Bac. Rev.*, 1949, v13, 135

13. Morgan, I. M., Howe, H. A., and Bodian, D. *Am. J. Hyg.*, 1947, v45, 379.

14. Morgan, I. M., *Am. J. Hyg.*, 1949, v49, 225.

The Lansing strain of poliomyelitis virus was used(15). The line§ used for vaccination had been in continued monkey passage and the pool had a titer of $10^{-2.5}$ PD_{50}. For neutralization tests 2 pools of the line¶ maintained in mice in this laboratory were used, with average LD_{50} titers of $10^{-4.0}$. Preparation and storage of the virus suspension has been described(16).

Four monkeys (18-61, -63, -65, -84) in individual cages were vaccinated with Lansing virus IIIa, while 4 were maintained as controls. Before each inoculation the animals were anesthetized with ether and blood samples of 5 cc were drawn from a femoral vein. After intracerebral challenge 20 to 40 cc of blood were drawn from the heart at each bleeding. The sera were stored at 4°C. Three (18-61, -63, -65) received 5 biweekly intravenous injections‖ of 4 ml of 5% virus suspension, representing a total intravenous inoculation of 1.0 g of infected nervous tissue. Each of these, and monkey 18-84, then received a total of 1.4 g of infected cord tissue intramuscularly, in doses of 2 ml of 10% suspension inoculated into the calf muscles once a week for 7 weeks. Intracerebral challenge with 1.0 ml of 10% virus suspension (140 PD_{50}) was given to 18-84 two weeks after the end of the course of vaccination, and to the others 3 weeks after the end of the course.

Monkey stools were collected daily and stored individually at –2 to –10°C. Each pool of feces represented 5 to 7 days' collection, except for the last 2 collections of 2 days each, from individual monkeys. A 10% suspension in physiological salt solution was prepared from each pool and kept at 4°C while centrifuged first at low speeds and finally for 1 hour at 15,000 r.p.m. (approx. 18,000 g) in an anglehead centrifuge(17). The final clarified supernate was kept at –2 to –10°C during precipitation with cold ethanol, according to methods used by Felton(18) and others(19). Cold 95% ethanol was added dropwise with constant agitation to yield a final ethanol concentration of 30%; frequently ethanol was added to another aliquot to yield a final concentration of 20% or 40%. The precipitate was removed by centrifugation (approx. 1450 g), and resuspended in 1-2 cc of supernate, dried in vacuum, and stored at –2 to –10°C. The efficiency of the alcohol procedure is indicated by the following considerations: 1), in one experiment, an average of 74% of the Lansing virus neutralizing antibody was recovered in the precipitate resulting from alcohol treatment of 7 human sera with original titers of 5 to 133 ND_{50} against 40 LD_{50} of the virus; 2) in another experiment, when 2 cc of human serum with a titer of 200 ND_{50} against 100 LD_{50} of virus was added to 50 cc of normal monkey stool suspension, over half of the virus neutralizing activity of the serum was recovered in the precipitates obtained with alcohol treatment, while simultaneous treatment of the same serum mixed with physiological salt solution resulted in a 60% recovery of the neutralizing activity of the unaltered serum. In addition, the pH values of the crude stool suspensions, as well as of the resuspended precipitates, were found to range from 6.1 to 7.5, in which range there is essentially no deleterious effect on immune globulins. In addition to tests of feces from immunized monkeys, stools were tested from

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15. Armstrong, C., *Pub. Health Rep.*, 1939, v54, 1719.

§ Received from Dr. Isabel Morgan, Poliomyelitis Research Center, Johns Hopkins University, Labeled "Lansing-Monkey IIIa".

¶ Received from Dr. S. D. Kramer in 1941.

16. Brown, G. C., and Francis, T., Jr., *J. Exp. Med.*, 1945, v81, 161.

‖ Immediately after the second or third and succeeding intravenous injections the monkeys developed respiratory arrest, accompanied by a rapid, thready, or imperceptible femoral pulse and cardiac thrust. This was treated by artificial respiration for 2 or 3 minutes, by which time the animals once more began to breathe, at first irregularly with gasping, and later rapidly and deeply with rapid recovery of consciousness. These incidents had no permanent ill-effects, and none was observed after intramuscular inoculation.

17. Pickels, F. G., *Rev. Scient. Instr.*, 1942, v13, 93.

18. Felton L. D., *J. Immunol.*, 1931, v21, 357.

19. Cohn, E. J., *et al.*, *J. Am. Chem. Soc.*, 1946, v68, 459.

four paralyzed poliomyelitic patients and two normal persons, all of whose sera had antibody titers of 600 to 2000 against 10 LD_{50} of Lansing virus.

The contents of each vial of dried material recovered by precipitation from the stool specimens were resuspended in 1.0 cc of physiological salt solution. The suspensions were tested in final dilutions of ½, ¼, 1/8, and 1/16 against final concentrations of virus suspensions of 5 or 1% at first (400 or 80 LD_{50}) and later against 0.5 and 0.15% (40 and 10 LD_{50}). Equal volumes of diluted serum, serum precipitate, or stool precipitate, were mixed with virus, incubated for 30 minutes at 37°C, and stored at 4°C for 30-90 minutes before inoculation. A volume of 0.03 cc of the mixture was inoculated intracerebrally into each of 8 mice lightly anesthetized with ether. A complete virus titration accompanied every test except for a few of the initial experiments with stool preparations in which a control was employed only for the infectivity of the virus concentration used. For all titrations and neutralizations, 50% endpoints were calculated according to the method of Reed and Muench(20). Neutralization results were expressed as reciprocals of the final dilution of serum giving that endpoint. Neutralization indices represent the product of the reciprocal of the final dilution of serum giving a 50% endpoint and the number of LD_{50}'s neutralized.

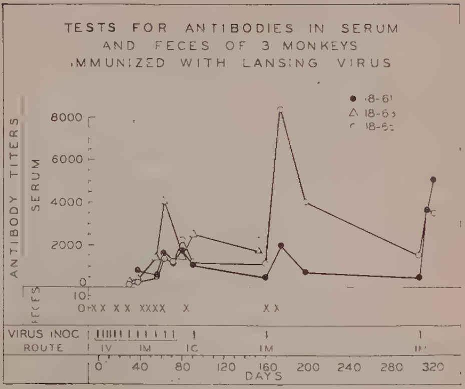

FIG. 1.

Results. Immunization of Monkeys. Results obtained with 10 LD_{50} of virus will be considered here, although most sera were also tested using 400 and 100 LD_{50} (Fig. 1). As seen in Fig. 1, virus neutralizing antibodies appeared in the serum of 4 monkeys after the twenty-first day. In three monkeys (18-61, -63, -65) antibody levels ranging from 1000 to 4000 were found in the blood towards the end of the 71-day period of immunization (Fig. 1). The antibody titer in the serum of monkey 18-84 rose to 813 two weeks after the end of the 56-day intramuscular course. A challenging intracerebral inoculation of 1.0 ml (140 PD_{50}) of the same 10% virus suspension used for vaccination was given the latter monkey two weeks after, and to the 3 others, (Fig. 1) whose sera maintained antibody levels of 1000 to 2500, three weeks after the end of the course of vaccination. Monkey 18-84, which had been vaccinated with a total of only 1.4 g of tissue (2000 PD_{50}) given intramuscularly and had the lowest titer of serum antibody, became paralyzed after the intracerebral challenge inoculation, as did 4 control animals which included 18-64 and 18-70. Histological study of the spinal cord confirmed the diagnosis of poliomyelitis in each case. The other 3 animals, each of which had been vaccinated with a total of 2.4 g of tissue (3400 PD_{50}) given by both the intramuscular and intravenous routes, developed no fever or other signs of poliomyelitis (Fig. 1). Approximately two-fold drops of serum antibody titer were noted in 2 of the 3 surviving immunized animals 10 weeks after the challenge inoculation. One of them (18-63) died at this time from a hemopericardium and showed no histological evidence of poliomyelitis. Immediately after this bleeding an additional inoculation of 0.2 g of the same material was given intramuscularly to the 2 survivors, and 2 weeks later the antibody titers were 1900 and 8300, respectively, thus surpassing their previous peak levels. Progressively lower values were obtained with blood taken 5 and 21 weeks after the booster dose in each animal, demonstrating that the antibody levels 21 weeks after the booster were as low as those obtained just

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20. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

before the booster. A second booster dose given 21 weeks after the first resulted in a 2 and 10-fold rise in titer to values of 3400 and 5100, respectively, within 2 weeks.

Antibody in the feces. The precipitates obtained with 20, 30 and 40% alcohol treatment of the stools collected from 4 vaccinated and 2 normal monkeys throughout the first 25 weeks of the test were consistently negative for the capacity to neutralize 10 or 40 LD_{50} of the Lansing strain of poliomyelitis virus. Using the technic of precipitation of stool extracts with alcohol in the cold, negative results were also obtained in tests with stools from two normal persons and four paralyzed poliomyelitic patients each of whose serum had antibody titers of 600 to 2000 against 10 LD_{50} of Lansing virus.

Discussion. Some observations were made concerning the development of antibody in the serum and of resistance to intracerebral challenge in vaccinated monkeys. The animal which received a total of 1.4 g of tissue (2000 PD_{50}) in 8 weeks was not immune to intracerebral challenge (140 PD_{50}) on the tenth week, when the neutralization index of the serum had reached 800. The other 3 monkeys received 2.4 g (3400 PD_{50}) in 10 weeks, and were immune when challenged intracerebrally in the thirteenth week at which time the neutralization indices of their sera were 10,000, 11,000 and 25,000 respectively. Additional inoculations on 2 subsequent occasions resulted in rises of antibody titer to levels considerably higher than those previously attained. The results of vaccination are similar to those reported by Morgan in extensive studies with the same strain of virus in monkeys(13,14).

There are several possible explanations for the failure to demonstrate Lansing antibody in the feces of immunized monkeys. One is that the procedures employed for its detection were not sufficiently sensitive. In this regard the evidence previously presented demonstrates the effectiveness of the method used: there was an average recovery of 74% of the neutralizing antibody for Lansing virus in precipitates resulting from alcoholic treatment of 7 human sera; there was approximately the same recovery of neutralizing substances by alcoholic precipitation of a mixture of serum and stool as by precipitation of a mixture of the same serum with saline. These results indicate that when specific antibody is present, in moderate amounts, at least, in serum or feces it can readily be detected by the methods employed. These methods might, of course, not be adequate for the demonstration of antibody if only a very small amount entered the intestine, especially since there is no known mechanism which would serve to concentrate antibody in the intestine. It is, therefore, conceivable that coproantibody might be detected if concentrates of the total feces excreted over a longer period of time were tested, rather than testing only the concentrates of smaller amounts as was done in the present experiments.

Another explanation for the absence of demonstrable coproantibody is that it was present but was combined with fecal components so as to lose its identity. The control studies mentioned in the preceding paragraphs demonstrate that when the specific antibody in the form of whole serum is added to feces it is neither destroyed nor removed by adsorption onto particulate fecal material discarded in the procedure. Or possibly antibody could be specifically absorbed by virus in the intestinal tract; this seems unlikely since virus was not demonstrable at any time in the alcoholic precipitates of fecal extracts. Failure to detect virus does not seem attributable to the procedures employed, since the Lansing strain of poliomyelitis virus is not inactivated, but on the contrary is concentrated, by precipitation with 20 to 30% ethanol(21). Similar concentration of the MEF strain of Lansing-type poliomyelitis virus has been reported by the use of methanol (22).

A third explanation for the failure to demonstrate coproantibody is that it was absent. This is the most likely reason for the present results although it may be that the antibody

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21. Unpublished data.

22. Pollard, M., Connolly, J., and Fromm, S., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 290.

titer of the serum has to be above the levels attained in the present experiments before a detectable quantity will be found in the feces. A trend toward such a threshold effect has been reported when the antibody to Lansing virus is found in nasopharyngeal secretions(4). But in the present experiments there was no evidence of this even when marked secondary rises in the antibody level of the serum were obtained.

It will be recalled that no neutralization of Lansing virus was demonstrable with extracts of feces from humans with moderate levels of antibody to Lansing virus in their sera. Morgan has also stated, without experimental details(23), that no neutralization of Lansing virus was obtained with the feces of 3 humans who had high titers of antibody in their sera. Although the response of human subjects to active infection might differ from that of monkeys to active immunization, the absence of coproantibody in both species suggests that the results obtained with the monkey may apply to man. Moreover, the antibody to Lansing in the serum of man is most probably acquired through earlier infection. Further studies with human subjects are, however, necessary to establish the absence of homologous coproantibody during subclinical or frank infection with poliomyelitis virus. One approach is indicated by a recent study of paralytic cases and their familial associates which suggests that the disappearance of poliomyelitis virus from the feces is related to the appearance or increase of homologous antibodies in the serum(24). Hammon has recently stated that feces from monkeys paralyzed with the MEF-1 strain of poliomyelitis virus neutralize the Lansing strain of virus(12). Since no experimental details are given a comparison of methods cannot be made. However, because of the present results one would assume that the effect reported by Hammon was probably related to something other than antibody unless the response of monkeys to active infection with the MEF-1 strain differs sufficiently from their response to immunization with the Lansing strain to account for the presence of coproantibody during paralysis.

No capacity to neutralize Lansing virus was demonstrated with extracts of pooled stools from each of 4 normal rhesus monkeys, nor with 11 pools from each of 2 other normal rhesus monkeys. These are similar to results mentioned by Hammon(12), but contrast with a report by Levaditi *et al.*(25) in which it was concluded that some neutralization of a monkey-adapted poliomyelitis virus was obtained with the stools of normal monkeys. However, Levaditi used cynomolgus rather than rhesus monkeys, and tested only crude stool suspensions.

Summary and Conclusion. Four monkeys were vaccinated with active preparations of the Lansing strain of poliomyelitis virus by parenteral routes. One animal received a total inoculum of 1.4 g of infected tissue, at the time of intracerebral challenge had an antibody titer in the serum of 800 against 10 LD_{50} of virus, but succumbed to an acute poliomyelitic infection. The remaining 3 received 2.4 g of inoculum, had antibody titers in their sera of approximately 2000 against 10 LD_{50} of virus and did not develop the disease. Each of 2 additional inoculations resulted in an increase in antibody of the serum to levels of 5100 and 8300 against 10 LD_{50} of virus. Despite the fact that Lansing virus antibody in the serum of three monkeys reached high titers, no evidence was obtained for the presence of antibody in the feces. Antibody was not detected in single specimens in the feces obtained from 6 humans whose sera had moderate titers of antibody to the Lansing strain of poliomyelitis virus. It is concluded on the basis of these data that antibody in feces is not an important factor in the resistance of the rhesus monkey, and probably of man, to infection with poliomyelitis virus.

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23. Morgan, I. M., *Poliomyelitis*, J. B. Lippincott Co., Philadelphia, 1948, p264.

24. Brown, G. C., and Ainslie, John D., in press. (Abstracted in *Fed. Proc.*, 1950, v9, 378.

25. Levaditi, C., Kling, C., and Lepine, P., *Bull. Acad. Med.*, Paris, 1931, v105, 190.

Received October 25, 1950. P.S.E.B.M., 1950, v75.

Adsorption of Protective Antigen of Hemophilus Pertussis on Human Red Cell Stromata.[*] (18311)

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Experiments directed toward the isolation of the protective antigen of *H. pertussis* have shown that this factor can be adsorbed quantitatively, selectively and without loss of potency on human red cell stromata.

H. pertussis (phase I) are grown in a modified Cohen and Wheeler fluid medium(1). The harvested bacteria are disintegrated in a 9 KC Raytheon sonic oscillator at 1° for periods of 15 minutes to 2 hours and the debris removed by centrifugation. The resulting extracts are added to appropriate amounts of red cells, of stromata, or of autoclaved stromata, incubated at 37°, centrifuged at 0° and the supernate discarded. The red cells are lysed with 50 volumes of water and the stromata removed by centrifugation. Details of the methods will be published at a later date.

The stromata protect mice in the intracerebral challenge test(2). Typical experiments are summarized in Table I which show that the protective doses (PD_{50}) of the stromata are equal to those of the bacterial suspensions from which they were prepared, while untreated stromata have no protective properties. Stromata do not adsorb pertussal toxin. They remove only a slight amount of nitrogen from the bacterial extract and cause little or no discernible change in the electrophoretic pattern of the extract. The removal of the protective antigen is therefore a complete and highly selective process.

TABLE I. A Comparison of the Protective Doses of Bacterial Vaccines and Stromata-Protective Antigen Complexes Prepared from *H. pertussis* (Phase I).

	Immunizing dose of material in billions of bacteria or their equivalent*			
	2.0	0.5	0.125	Protective
Sample	S/T	S/T	S/T	dose$_{50}$†
Bacterial vaccine No. 138	10/10	9/10	7/10	<.125
Adsorbed stroma No. 138	9/10	8/10	7/10	<.125
Bacterial vaccine No. 134	9/10	7/10	2/10	.310
Adsorbed stroma No. 134	8/10	7/10	2/10	.342
Stroma	0/10	0/10	0/10	—

S = No. of survivals.
T = No. of animals challenged.

National Institutes of Health Reference Vaccine #4 had a PD_{50} of 0.332 when tested with #138 and 0.449 when tested with #134.

* Samples were diluted with 0.9% sodium chloride to contain the immunizing dose in 0.5 ml. Suspensions of adsorbed stroma were adjusted to be equivalent to the bacterial vaccines.

† Calculated by the method of Reed and Muench.

It has been suggested that the protective potency of culture filtrates and bacterial vaccines of *H. pertussis* are related to their hemagglutinin content(3). Although the hemagglutinin of the supersonic extracts is also adsorbed on stromata, we believe that the hemagglutinin and protective antigen are not identical. No correlation between hemagglutinin titers and protective potency of either bacteria or extracts has been observed in this laboratory. A culture filtrate of 72 hours growth of *H. pertussis* contained as much hemagglutinin as the supersonic extracts. The hemagglutinin was completely adsorbed on stromata but gave no protection in the intracerebral challenge test. Supersonic extracts heated to 56° for 1 hour retain their hemagglutinating and protective power. However, while the hemagglutinin is still adsorbed completely on stromata, such stromata offers little or no protection. Furthermore, certain strains of *H. pertussis* have no hemagglutinin content and are excellent sources of protective antigen. These observations strongly suggest that the hemagglutinating and protective activities of the extracts reside in distinct chemical structures. Attempts are now being made to elute the protective antigen from stromata. It is hoped that such experiments may help to clarify this problem.

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The stromata-antigen complex is a highly potent protective agent as judged by the Kendrick test(2); it is free of pertussal toxin and represents only a very minute portion of the supersonic extracts. The complex may therefore be an improved prophylactic agent against whooping cough. Since group O-Rh negative red cell stromata may be used for the adsorption of the protective antigen, this carrier should offer no clinical disadvantage, but may serve as an important stabilizing adjuvant. The clinical efficacy of the stromata-antigen complex will need to be proven by extensive field trials which are now being planned.

Summary. The protective antigen of *Hemophilus pertussis* has been adsorbed quantitatively, very selectively, and without loss of potency on human red cell stromata. The protective antigen and the hemagglutinin of *Hemophilus pertussis* are not identical. Clinical application of the stromata-protective antigen complex is discussed.

The author wishes to acknowledge the able technical assistance of Mrs. Livia Blum and Mrs. Eleanor Brown. Thanks are due to Dr. J. N. Adam, Jr., and Miss Frances L. Clapp for supplying some of the bacterial suspensions employed in this study and for carrying out the intracerebral challenge tests.

Received October 27, 1950. P.S.E.B.M., 1950, v75.

Porphyrins in the Bone Marrow and Circulating Erythrocytes in Experimental Anemias.* (18312)

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Recent reports(1-3) have described the occurrence of coproporphyrin as well as protoporphyrin in the circulating red blood cells, and have pointed to the correlation of the former compound with erythropoietic activity as revealed by reticulocyte percentage. These studies have induced us to examine the porphyrin content of the bone marrow in certain experimental anemias in rabbits, and to compare it with that of the circulating erythrocytes when examined simultaneously. Very little has been reported in the previous literature bearing on this matter. Vannotti(4) was able to extract considerable amounts of protoporphyrin from the bone marrow of rabbits with experimental hemorrhagic anemia. He also remarked upon the presence of coproporphyrin in the bone marrow of lead poisoned rabbits. He thought that this was derived from protoporphyrin, although there was no supporting evidence for this belief. DeLangen and Grotepass(5) observed small amounts of copro-and larger amounts of protoporphyrin in the bone marrow of a horse poisoned with lead. Stasney and McCord(6) found increased amounts of porphyrin (type not identified) in the bone marrow of cases of iron deficiency anemia and

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* Aided by grants from the U. S. Public Health Service, Division of Research Grants and Fellowships (RG-345), and the Atomic Energy Commission, Contract Nos. AT (11-1)-100 and AT (11-1)-108.

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TABLE I.

Rabbit Nos.	Details of experiments
51, 44, 57	Normal controls
5, 7	100 mg lead acetate 15 and 4 days (No. 5) and 28 and 7 days (No. 7) prior to examination*
13, 15, 16, 18, 21	A single inj. of 100 mg lead acetate/kg body wt 4 wk (No. 13, 15, 16) and 5 wk (No. 18, 21) prior to examination*
6, 8	225 ml (No. 6) and 212 ml (No. 8) of blood removed in repeated bleedings over a period of 5 wk (No. 6) and 4 wk (No. 8)
22	Exposed continually in air to atmospheric pressure of 320 mm Hg (corresponding to 23,300 feet altitude) for 5 days prior to examination
9, 10, 23	130 mg (No. 9), 180 mg (No. 10), 160 mg (No. 23) phenylhydrazine in 3 or 4 divided doses over 8 to 10 days prior to examination†

* The lead acetate was dissolved in water with a few drops of buffered glacial acetic acid to bring the pH to approximately 5.5. The final sol. containing 25 mg of lead acetate per ml was administered by subcut. inj.

† Phenylhydrazine hydrochloride, dissolved in isotonic sodium citrate sol. was given in each case by subcut. inj.

pernicious anemia in remission, while minimal amounts were observed in pernicious anemia in relapse, aplastic anemia, leukemia, and sprue. The method of estimating amount was simply visual, depending on intensity of red fluorescence in the extracts.

The purpose of the present communication is to record the results of determinations of copro- and protoporphyrin in bone marrow and circulating red blood cells of presumably normal rabbits and rabbits subjected to 1) lead poisoning, 2) bleeding, 3) phenylhydrazine intoxication, and 4) reduced oxygen tension.

Methods. Adult rabbits of either sex were used. Seven were given lead, 3 phenylhydrazine, 2 were bled, and one was exposed to reduced oxygen tension. Further details of these experiments are given in Table I. At the conclusion of each experiment the rabbit was bled to death by cardiac puncture, after intravenous injection of 2 cc of heparin. This blood was employed for porphyrin determinations. Immediately after death both femora and humeri were freed and removed, the marrow cavities opened, and the bone marrow spooned out and suspended in isotonic sodium citrate solution. This was shaken for 1 minute to reduce trapping of cells between the marrow fat particles. The tube was then centrifuged for 8-10 minutes at 1500 r.p.m. after which the supernatant layer, including the fat was pipetted off. The packed cells were then resuspended in sodium citrate solution and small portions of this suspension were used for morphologic study, differential counts, and determinations of the percentage of nucleated red blood cells. The suspension was then spun down at 2000 r.p.m. for 30 minutes, the packed cell volume measured, and the copro- and protoporphyrin determined according to the method of Schwartz and Wikoff(2).

Results. The important data are shown in Table II. It is seen that in the normal rabbits the concentration of BMCP (bone marrow coproporphyrin) is only slightly greater than that of the ECP (erythrocyte coproporphyrin), and that protoporphyrin concentration is approximately the same in the bone marrow and circulating erythrocytes. In all four groups of experimental rabbits, on the other hand, BMCP values are markedly elevated, while levels of BMP (bone marrow protoporphyrin) are nearly all lower than those of the corresponding EP (erythrocyte protoporphyrin).

Certain differences between the experimental groups are apparent in the relative concentrations of the two porphyrins in bone marrow and peripheral blood as summarized in Table II. Thus the lead-poisoned rabbits show the greatest relative increase of BMCP as compared to ECP, while both BMP and EP are markedly elevated. The data of the

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TABLE II. Summary of Hematological and Porphyrin Data.

Group	Rabbit No.	% of nucleated RBC in BM*	% of non-nucleated RBC in BM†	BMCP γ%	BMP γ%	ECP γ%	EP γ%	% of reticulocytes in peripheral blood	Hb in g per 100 ml blood
Normal	51	31	—	1.7	71.4	0.6	58	1.2	12.7
	44	49	—	3.2	54	0.4	106	2.1	13.1
	57	49	—	8.6	137	0.4	86	2.5	12.3
Low O_2 tension	22	67	—	42	90	2.1	246	7.6	16.2
Bleeding	6	41	—	52	186	4.1	237	4.4	8.7
	8	55	23	67	148	15.9	393	15.8§	7.1
Phenylhydrazine	9	73	9	75	111	167	221	85 §	6.5
	10	87	—	99	163	102	441	55 §	6.5
	23‡	54	—	89	96	3.9	88	87 §	7.9
Lead	5	62	17	189	450	5.8	615	12.6	7.2
	7	49	—	194	226	6.0	647	17.4	8.1
	13‡	62	36	32	63	1.0	425	3.0	12.3
	15	39	43	409	454	3.1	402	2.4	8.6
	16	45	38	240	1296	0.3	534	1.0	12.5
	18	38	62	54.5	712	1.1	361	1.3	10.6
	21	35	24	92.5	420	1.3	505	3.2	10.5

* These values were calculated as percentage of total nucleated (myeloid-erythroid) bone marrow cells.

† These values were calculated as percentage of total nucleated and non-nucleated (myeloid erythroid) bone marrow cells.

‡ These rabbits differed from the others in being unusually large and old.

§ Frequent normoblasts in peripheral blood.

rabbits with post hemorrhagic anemia and low oxygen tension are similar though at a lower level of porphyrin concentration.

In the rabbits receiving phenylhydrazine, the findings are of unusual interest because of the marked elevations of ECP and, to a relatively lesser extent, of the EP in two of the three animals. As noted in Table II, many normoblasts were seen in the stained blood smears of these rabbits. The high ratio of ECP to EP agrees with previously reported data(1,2).

As shown in Table II, it is clear that a variable number of mature erythrocytes are present in the bone marrow. While no attempt has been made to apply a correction factor for these cells, it is obvious that their presence does not affect the general conclusions to be drawn.

Discussion. The present findings offer further evidence pointing to a close relationship of coproporphyrin to hemoglobin synthesis. Two divergent concepts regarding this relationship have recently been advanced. One is that coproporphyrin III is a normal intermediary in the formation of the hemoglobin protoporphyrin(7,8). The other hypothesis (9) emphasizes oxidative decarboxylation of a dipyrrylmethene precursor of coproporphyrin to one of protoporphyrin. According to this, coproporphyrin is formed as a side product and is not a precursor of the hemoglobin protoporphyrin. It is believed that the present data are compatible with either hypothesis. There is, however, one consideration which is more readily explained by the assumption that coproporphyrin is a direct precursor of hemoglobin protoporphyrin. This involves the correlation of previous data obtained for urinary and fecal coproporphyrin(1,10,11) with those now obtained for bone marrow and blood. In instances of heightened erythro-

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poiesis as seen in acute blood-loss anemia or in hemolytic (phenylhydrazine) anemia there is a considerable increase of total coproporphyrin in both bone marrow and circulating erythrocytes. Studies in this laboratory(2) revealed 90% of the total erythrocyte coproporphyrin to be the type III isomer in animals subjected to repeated bleedings, while approximately 60% was type III in animals with phenylhydrazine anemia. The major fraction of the red cell coproporphyrin thus has the same configuration as hemoglobin protoporphyrin. On the other hand, the increase in urinary and fecal coproporphyrin with increased erythropoiesis is due to the type I isomer, with little or no change in the type III coproporphyrin(1,10,11). This is consistent with the concept that there is efficient utilization of coproporphyrin III for protoporphyrin synthesis, with a liberation from the cells of increased amounts of coproporphyrin I formed as a side product. However, if coproporphyrin III also were only a side product, one might expect that it, too, would be excreted in increased amount. As noted, this is not the case though it can be argued that other unknown factors may operate to influence the excretion or further metabolism of coproporphyrin III.

The data obtained in the lead poisoned animals agree equally well with either hypothesis. Thus, Lemberg and Legge point out that their scheme would predict the increase in type III isomer which is known to exist. According to the precursor concept, too, it would be logical to assume that lead interferes with the utilization of coproporphyrin III; hence its increased excretion in the urine. The evidence also suggests interference by lead in other stages of hemoglobin synthesis, *i.e.* in the utilization of protoporphyrin as indicated by its accumulation in marrow and circulating cells, and perhaps of porphyrin precursors. In this connection it is of interest to point out that the porphyrinuria of lead poisoning is readily harmonized with the present bone marrow findings while previous studies of the circulating erythrocytes(2,3) failed to reveal any correlation of the coproporphyrin concentration in urine and erythrocytes. The data suggest that the coproporphyrin is liberated from intact young erythrocytes in the bone marrow. Actual destruction of these cells appears unlikely since the excesses observed in the rabbits urine would require the destruction of 100 ml or more of such cells per day.

Despite the evident relationship of coproporphyrin to erythropoiesis, it may be pointed out that the present investigation has not revealed any strict correlation between the degree of erythropoietic activity (percentage of normoblasts) in the bone marrow, and its coproporphyrin concentration nor between the percentage of circulating normoblasts and the coproporphyrin concentration of the circulating erythrocytes.

An ether-insoluble uro-type porphyrin has also been demonstrated in the bone marrow of lead poisoned rabbits. Its absorption and fluorescence spectra are identical with those of uroporphyrin I. Further studies of this porphyrin are in process.

Summary and conclusions. 1. The copro- and protoporphyrins of bone marrow and circulating erythrocytes in the rabbit have been found to exhibit dynamic changes under a variety of stimuli to erythropoiesis, including lead poisoning, phenylhydrazine, hemorrhage, and reduced oxygen tension. These consist of (1) a striking increase of coproporphyrin in the developing erythrocytes of the bone marrow, with a marked relative reduction of its concentration in circulating red blood cells, (2) an inconstant but generally increased level of protoporphyrin in the circulating as compared with the marrow erythrocytes. 2. These observations point to a close relationship between coproporphyrin and hemoglobin synthesis, and appear to be consistent with concepts which consider coproporphyrin either as a direct precursor of hemoglobin protoporphyrin or as a by-product of the synthesis. The former concept, however, seems to permit a more reasonable correlation of the porphyrin findings in bone marrow and blood with those in urine and feces.

Received October 30, 1950. P.S.E.B.M., v75.

Response of Pigs to Streptomycin.* (18313)

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Using *ad libitum* feeding Moore *et al.*(1) found that streptomycin produced a growth response in chicks fed a casein-synthetic type ration. Stokstad *et al.*(2) using a semi-practical ration supplemented with vitamin B_{12} found that a growth response by chicks was obtained from a fermentation product of *Streptomyces aureofacians* and a similar growth response was obtained from crystalline aureomycin hydrochloride. Jukes *et al.*(3) reported that aureomycin-hydrochloride added to a corn-peanut basal ration supplemented with vitamin B_{12} produced approximately a 50% increase in average daily gain of weanling pigs and a reduction in the incidence of diarrhea. Luecke *et al.*(4) reported similar results with streptomycin when added to a corn-soybean meal ration.

Experimental. Four pairs of 2-day-old pigs were used to study the effect of adding streptomycin to a complete "synthetic milk" ration for baby pigs. The basal ration was the 30% alpha protein "synthetic milk" previously described by Neumann *et al.*(5). Vitamin B_{12} was injected intramuscularly at the rate of 0.8 μg per kg of body weight per day. Two grams of sulfathalidine were added per liter of milk. The technic of care and feeding of the pigs has been described by Johnson *et al.*(6). The pigs were pair-fed equal feed intakes for 7 weeks. One pig of each pair received the basal diet, while the other received the basal diet plus 500 mg of crystalline streptomycin per kg dry matter. The results are summarized in Table I.

TABLE I. Effect of Streptomycin on Growth and Feed Utilization of Baby Pigs (Pair-fed for 49 days).

	Basal diet	Basal + streptomycin
Avg initial wt, kg	1.50	1.51
Avg final wt, kg	16.54	18.27
Avg daily gain, kg	.306	.342*
Live wt gain/kg dry matter consumed, kg	.71	.80*

* Highly significant over the basal diet. $P<0.01$.

Summary. The addition of 500 mg per kg dry matter of streptomycin to a nutritionally adequate "synthetic milk" containing 2 g of sulfathalidine per liter produced a highly significant increase in the average daily gains and a highly significant reduction in the amount of dry matter required per kilogram body weight gain.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for streptomycin and water soluble vitamins; to Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for folic acid; and to Sharp and Dohme, Inc., Glenolden, Pennsylvania, for sulfathalidine.

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Received October 30, 1950. P.S.E.B.M., 1950, v75.

Effect of Antibacterial Agents on Growth of Baby Pigs Fed a "Synthetic" Diet. (18314)

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The effect of antibiotics in promoting the growth of pigs was first reported by Stokstad and Jukes(1), and by Johnson(2). The growth-promoting effect of aureomycin when added to the diet of weanling pigs has been reported by Jukes *et al.*(3). Carpenter(4) has reported less scouring and also some growth stimulation when feeding aureomycin to growing pigs.

The present study was conducted to determine the growth-stimulating effect of aureomycin, penicillin and sulfathalidine on the baby pig receiving a "synthetic milk" diet supplemented with all known factors.

Experimental procedure. Thirteen 2-day-old Berkshire pigs were allotted into 5 groups and individually fed *ad libitum*. The alpha-protein "synthetic milk" ration fed was essentially the same as reported by Neumann *et al.* (5). In addition 0.8 μg of vitamin B_{12} per kilo of body weight per day was injected intramuscularly. The feeding and care of the animals was similar to that reported previously by Johnson *et al.*(6). The levels of antibiotics and sulfathalidine fed are given in Table I. At periodic intervals fecal samples were collected from one pig in each of groups 1, 2, 4, and 5. Coliform, lactobacilli and yeast cell counts were determined on these samples by dilution methods employing EC medium (Difco) for the *E. coli* counts, Lactalysate broth (BBL) for the lactic counts and Winblad's acetic acid medium(7) for the yeast counts. Weighed samples of 0.1 g were diluted to 100 cc to give a concentration of 10^{-3}. Dilutions were then made from this concentration. The results are given in Table II.

Results and Discussion. A summary of the total performance of each of the 5 groups of pigs is given in Table I. The growth curves of each group are plotted in Fig. 1. Because of the small number of pigs in each group, Groups 1 and 2 receiving no aureomycin were combined for statistical treatment and compared with the combination of Groups 3 and 4 which received aureomycin. The average daily gain of the latter combination was significantly more rapid than the average daily gain of the former ($P = 0.03$). The 6 pigs receiving aureomycin had an average daily gain of over 1 lb/day for the 8-week experimental period. This is remarkable growth for pigs of this age.

The addition of 2% sulfathalidine or 100 mg of penicillin per kilo of dry matter of the diet did not increase 8-week gains significantly.

The bacteriological counts showed a decrease in the number of *E. coli* in the feces of the pigs receiving sulfathalidine, as has been previously observed for other species(8-10). Lactobacilli and yeast cell counts were high and apparently not affected by any treatment.

Summary. Aureomycin stimulated the growth of baby pigs on an alpha protein "synthetic milk" diet. It did not reduce the coliform, lactobacilli or yeast cells present in the feces. Sulfathalidine reduced the num-

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TABLE I. Response of Baby Pigs to Antibiotics and Sulfathalidine.

	Group 1	Group 2	Group 3	Group 4	Group 5
	Basal	Basal + 2% sulfathalidine	Basal + 2% sulfathalidine + 100 mg aureomycin/kg dry matter consumed	Basal + 100 mg aureomycin/kg dry matter consumed	Basal + 100 mg penicillin/kg dry matter consumed
No. of pigs	2	2	3	3	3
Avg initial wt, kg	1.83	1.81	1.78	1.76	1.74
Avg final wt, kg	20.57	20.40	28.86	26.40	21.51
Avg daily gain, lb.	0.74	0.73	1.06	0.97	0.78
Dry matter consumed (kg/kg gain)	1.30	1.34	1.29	1.32	1.28

TABLE II. Bacteria and Yeast Cell Dilution Counts Giving Greatest Dilution at Which Growth Occurred.

Days on test	Group 1 Basal			Group 2 Basal + 2% sulfathalidine			Group 4 Basal + 100 mg. aureomycin/kg dry matter consumed			Group 5 Basal + 100 mg penicillin/kg dry matter consumed		
	E. coli	Yeast	Lactic	*E. coli*	Yeast	Lactic	*E. coli*	Yeast	Lactic	*E. coli*	Yeast	Lactic
1	10^{-8}	10^{-6}		10^{-6}	10^{-6}		10^{-6}	10^{-6}		10^{-6}	10^{-4}	
3	10^{-11}	10^{-8}		10^{-8}	10^{-10}		10^{-11}	10^{-6}		10^{-11}	10^{-6}	
5	10^{-11}	10^{-11}		10^{-9}	10^{-10}		10^{-11}	10^{-10}		10^{-10}	10^{-8}	
8	10^{-9}	10^{-8}		10^{-6}	10^{-10}		10^{-10}	10^{-10}		10^{-11}	10^{-6}	
12	10^{-11}		10^{-10}	10^{-6}		10^{-11}	10^{-11}		10^{-11}	10^{-11}		10^{-11}+
19	10^{-11}		10^{-11}	10^{-6}		10^{-10}	10^{-10}		10^{-11}	10^{-11}+		10^{-11}+
26	10^{-11}	10^{-8}	10^{-10}	10^{-6}	10^{-6}*	10^{-9}	10^{-10}	10^{-8}	10^{-10}	10^{-12}	10^{-8}*	10^{-11}

* Microscopic examination showed a few lactobacilli but yeasts were predominant.

ber of coliform bacteria present in the feces. There was no statistically significant beneficial growth-promoting effect from adding penicillin or sulfathalidine to the diet.

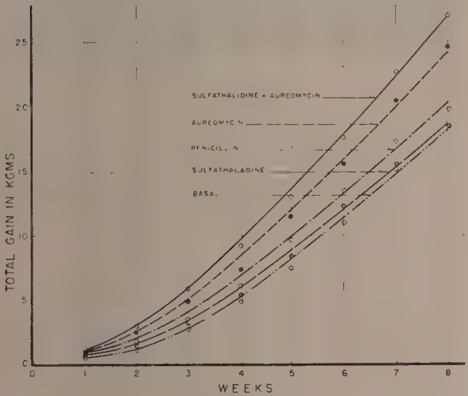

FIG. 1.

Graph showing the average growth responses of the various groups.

We are indebted to Merck and Company, Inc., Rahway, New Jersey for crystalline vitamins, and penicillin; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for folic acid and aureomycin HCl; and to Sharp and Dohme, Inc., Glenolden, Pennsylvania, for sulfathalidine.

We are also indebted to Eva M. Cohn for carrying out the bacterial counts.

Received October 30, 1950. P.S.E.B.M., 1950, v75.

Neurosecretion Following Hypophysectomy. (18315)

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Elucidation of the various factors controlling the pituitary gland has been a major problem of science for many years. Harris (1) has admirably reviewed the literature concerning the neural control of the hypophysis. His statement, 'The pars distalis of the pituitary may in general terms be described as a gland under nervous control but lacking a nerve supply," most aptly phrases one of the oldest problems of neuro-endocrinology, and the same statement may well be extended to parts of the hypothalamico-hypophyseal system other than the pars distalis. However, a careful survey and analysis of the literature reveals that in addition to a direct neural control via nerve fibers at least three additional controlling or influencing mechanisms are known or postulated. They are: (A) a hypothalamico-hypophyseal portal system(2-5); (B) the reciprocal reactions and related functions of parts of the hypophysis to the other endocrine glands (literature too numerous to list); (C) and last, the fact that secretory material elaborated by the hypothalamic neurons may pass to the hypophysis via neurons or by way of the perineuronal spaces(6,7).

Although Scharrer and co-investigators have thoroughly covered the morphological aspects of neurosecretion, extremely little or virtually no experimental observation has been undertaken in the field of neurosecretion. This is especially true in the vertebrates. Speidel(8) conducted some of the earliest experiments on neuro-secretion, using skates as an experimental animal, with negative results. The only positive evidence so far published regarding the relationship of neurosecretion to any other physiological processes of the vertebrate has been offered by Drager (9). A full report of this work with additional experimental evidence is in preparation.

In certain neuro-endocrine studies of the tropical indigo snake, *Spilotes corais*, the animals were hypophysectomized according to a method described previously(10), and following hypophysectomy the empty sella turcica was packed with fibrin foam. Animals so treated were sacrificed at 2, 3 and 4 weeks postoperatively. The fibrin foam and brain were removed, fixed, sectioned serially and stained by Foot's modification of the Masson method. In species of this animal sacrificed 3 weeks after hypophysectomy the supra-optic nucleus is not completely degenerated and contains a small amount of neuro-secretory material. Furthermore, in animals sacrificed at 2 and 3 weeks postoperatively, the fibrin foam within the empty sella contains an appreciable number of spherical granules. The staining characteristics of these granules are identical to the secretory granules within the nerve cells of the hypothalamus. On the basis of staining characteristics and following the comparison of these granules with the secretion from and within the nerve cells of the supra-optic nucleus, there can be little doubt that such granules come from the hypothalamic neurons and pass along the hypothalamico-hypophyseal nerve tract to be lodged in the fibrin foam within the sella (Fig. 1, 2, and 3).

As the granules pass toward the pituitary they maintain close relationship to the nerve

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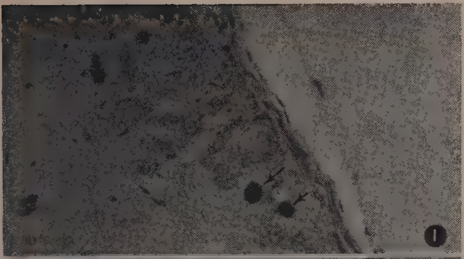

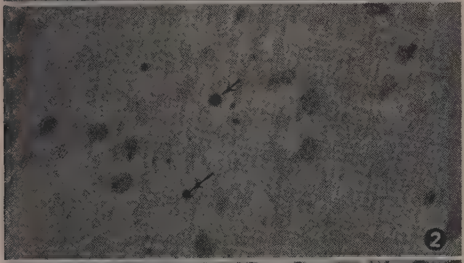

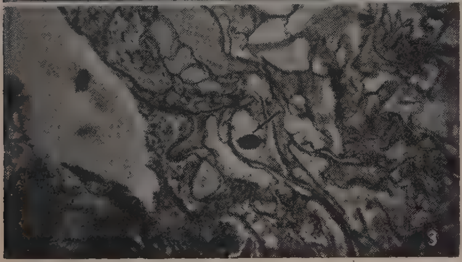

FIG. 1. Secretory material, indicated by arrows, within the supra-optic nucleus of the intact animal.

FIG. 2. Arrows indicate several secretory granules dispersed along the hypothalamico-hypophyseal nerve tract of the intact animal.

FIG. 3. Secretory granule lodged in the fibrinfoam.

fiber tract and apparently follow the perineural spaces to the hypophysis.*

It has been questioned whether the so-called "granules of Scharrer" were not merely artifacts of colloid which had migrated "up stream" along the hypothalamico-hypophyseal nerve tract.† This experiment strongly indicates that the "granules of Scharrer" from the magnocellular portion of the hypothalamus reach the hypophyseal region in the absence of the pituitary gland. Under conditions described it seems impossible that these granules can be upward migratory products from the hypophysis and therefore such secretions can hardly be the products of degeneration as once postulated. The foregoing statement is substantiated by Drager(9) when it was found that extirpation of all the endocrine glands had little or no effect on the hypothalamic neurons, yet hepatectomy invariably produced a marked decrease in the secretory granules and a vacuolation of the cells in the magnocellular portions of the hypothalamus.

Although the fact exists that secretory products from the hypothalamus traverse the hypothalamico-hypophyseal tract, few investigators of the hypophysis recognize this as a possible controlling mechanism of the pituitary gland. However, considerable evidence now points in this direction.

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* In the average (normal) animal the granules may be observed in the large cells of the hypothalamus, along the hypothalamico-hypophyseal nerve tract and in the posterior lobe of the pituitary. In the toad such granules are often present in the intermediate lobe of the hypophysis.

† Bargman and Held, investigating dogs and cats, have traced the secretion products of the supra-optic and para-ventricular nuclei to the pars nervosa. These investigators interpret the bodies of Herring as hypothalamic secretory condensations in the posterior pituitary. No experimental evidence is offered for their conclusions. *Acta. Anat.*, 1949, v7, 264.

Received October 30, 1950. P.S.E.B.M., 1950, v75.

Note on the Electrophoresis of Thrombin.* (18316)

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It has been possible to prepare prothrombin of sufficient purity to make it feasible to study its electrophoretic properties(1,2). Its isoelectric point is near pH 4.2 in buffer solution of 0.1 ionic strength, and the mobility is nearly the same as that of α-globulin. These electrophoretic properties of the protein change when it is activated to thrombin. This is most neatly demonstrated by dissolving the prothrombin in 25% sodium citrate solution. In this environment activation takes place by autocatalysis(2,3). There is no need to add such activators as calcium, thromboplastin, Ac-globulin, etc.; hence, upon activation there are no uncertainties introduced by these substances or by any impurities that might be associated with these activators. The products of prothrombin activation may thus be considered to be derived from prothrombin itself. Usually this has of necessity been considered only in terms of thrombin activity, but work with purified materials shows that more than one substance is derived from prothrombin as the result of its activation. In fact, at least three distinct entities are indicated by elecrophoretic analysis to be represented in thrombin preparations. These 3 substances need further investigation; but information gained from electrophoretic studies is reported in this paper. It was especially desired to determine the isoelectric point of the components in our thrombin preparations and to see whether it might be possible to make electrophoretic separations.

Experimental. The methods employed for the assay(4,5) and preparation of materials (1,2) have been described in detail in previous work. The prothrombin preparations did not have all impurities eliminated, because large quantities were needed in the macro-electrophoresis cells of the Tiselius apparatus and this made the cost of materials a factor to be considered. Consequently it seemed justifiable to compromise and work with prothrombin definitely known to contain impurity. The prothrombin was activated in 25% sodium citrate solution, precipitated with ammonium sulfate and freed of the latter by dialysis. All assays for activity were made as previously described.

Results. It was possible to follow the

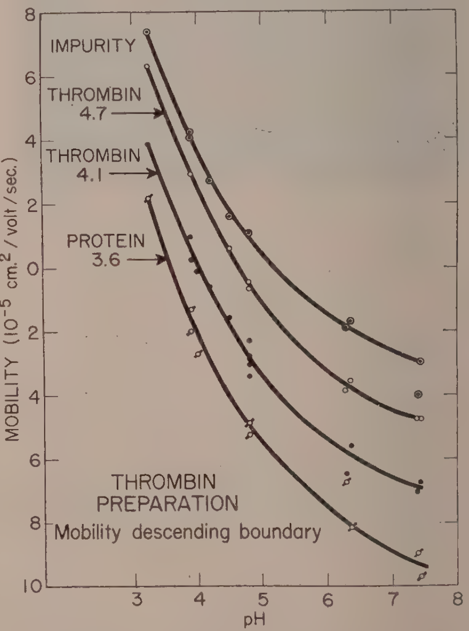

FIG. 1.

Electrophoretic mobility of purified bovine thrombin in barbiturate, phosphate, and acetate buffers of 0.1 ionic strength. The mobilities were calculated from observations of the descending boundary pattern.

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* Aided by a grant from the National Institutes of Health, United States Public Health Service.

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mobility of 4 components (Fig. 1). One of these could be considered impurity because it was also found in the prothrombin preparations from which the thrombin was derived. The other 3 components have their isoelectric points at about pH 4.7, 4.1 and 3.6. One of these values is near that found for prothrombin which, under the same conditions, has an isoelectric point of about pH 4.2. The latter value is somewhat uncertain because prothrombin is not sufficiently soluble at its isoelectric point to permit accurate measurement. The value represents the result of extrapolating a curve rather than precise determinations. Such solubility difficulties are not encountered with thrombin preparations. The protein with an isoelectric point of pH 4.1 is quite soluble at that pH. It is thus different from the protein found in the prothrombin preparations. Of the other 2 proteins, one has an isoelectric point at pH 4.7 and the other at pH 3.6. Tentatively we are referring to these proteins by the terms 4.7-Thrombin; 4.1-Thrombin; and 3.6-Protein. This conveys the idea that two possess thrombic activity and that the third one does not.

Analytical solubility curves described by Seegers and McGinty(6) and repeated again later indicated the possibility that thrombin activity might be possessed by 2 different proteins. In electrophoretic separation experiments this idea received further confirmation. However, the work could not be done as nicely as we had hoped. Consequently it is only possible to say that it is quite likely that thrombic activity resides in 2 proteins, that the question is not satisfactorily answered, and that there are no indications to the contrary. Work on the 3.6-protein was somewhat more encouraging. This component is present in low concentration but has a high mobility and it was possible to separate material which most probably represented this protein. It was free of thrombic activity and it contained much less tyrosine than the thrombin preparation as a whole. It contained carbohydrate. The separations in several experiments were considered to be technically satisfactory and it seems justifiable to conclude that this protein is inert with respect to thrombic activity.

Summary. Results of electrophoresis experiments with purified thrombin preparations indicate that thrombic activity is probably associated with 2 proteins. Under the conditions of the experiments one has an isoelectric point of pH 4.1 and the other at pH 4.7. Another substance is derived from prothrombin and is always found in the thrombin preparations. It has a high electrophoretic mobility and its isoelectric point is 3.6. This is called 3.6-protein, and it is very likely devoid of thrombic activity.

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Received October 30, 1950. P.S.E.B.M., 1950, v75.

Cortisone-induced Transformation of Ovaries into Testes in Larval Frogs.* (18317)

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In continuation of earlier experiments with standard androgenic and estrogenic substances, a study was made of the effects of some alkyl substituted androstanes on the process of sex differentiation in larval frogs *(Rana sylvatica)*. The 4 investigated compounds all brought about inversions in genetic females. However, the required dosages are high in comparison with testosterone and its derivatives(1).

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* Aided by grants from the National Research Council, Medical Division; Committee for Research in Problems of Sex. We are further indebted to Ciba Pharmaceutical Products, Inc. for the gift of Pregnenolone, Progesterone, and Desoxycorticosterone acetate.

TABLE I. Effect of Pregnenolone on Sex Differentiation in *Rana sylvatica.*

Dosage µg/l	Sex distribution		
	♂	⚥	♀
500	41		
100	41	1	
25	27		21
Controls	72		99

TABLE II. Effect of Progesterone on Sex Differentiation in *Rana sylvatica.*

Dosage µg/l	Sex distribution		
	♂	⚥	♀
500	58	10	
250	36	30	10
100	39	13	40
20	37	7	48
Controls	45	1	48

TABLE III. Effect of Desoxycorticosterone Acetate on Sex Differentiation in *Rana sylvatica.*

Dosage µg/l	Sex distribution		
	♂	⚥	♀
1000	65	14	
500	67	14	7
200	41	2	47
Controls	45	1	48

The substance to be tested is first dissolved in absolute alcohol, at near saturation. The desired concentrations in the water of the aquaria containing the larvae are obtained by "injecting" from a graduated syringe adequate amounts of this concentrate or of properly prepared alcoholic dilutions. Controls show that the small amounts of the solvent (0.05 to 0.25 ml per l of water) thus introduced, are without effects. Treatments usually are started at stage 25, when the gill sacs have closed and the gonads are still morphologically indifferent; they are continued until metamorphosis (stages 30-33).

Pregnenolone at 100 µg and 500 µg per liter causes the complete masculinization of all genetic females, while 25 µg/l remain without effect (Table I). It may be concluded that the critical or threshold concentration is at about 50 µg/l.

Progesterone has considerable anesthetic and toxic qualities, especially if administered at early stages. Partial masculinization of the female begins at 250 µg/l. At 500 µg/l, the sublethal concentration, the masculinization of all females is practically complete (Table II).

Desoxycorticosterone acetate is less toxic than progesterone though, as in the latter, masculinizing effects appear only at concentrations that approach the lethal limit. At 500 µg/l not all ovaries are definitely affected; but at 1000 µg/l all females have become transformed into hermaphrodites or males by the time of metamorphosis (Table III). This result is not in agreement with a report by Vannini(2) who contends that solutions of 400 µg/l in *R. dalmatina*, effect a reduction of the medullary component of the ovaries. According to our observations on *R. sylvatica* one should not expect any pronounced reaction with this dosage.

Cortisone became available to us late in spring, when the youngest of this year's sylvatica larvae had already entered the stage of sexual differentiation (stage 25/26). A group of 20 was placed in a concentration of 1 mg/l. The slightly late start of the treatment may account for the initial establishment of the normal sex ratio (11 ♀ + 9 ♂). When after 25 days of the treatment 3 larvae were preserved, it was found that 2 of this group were females. Their ovaries show first, but unmistakable traits of sex inversion (Fig. 1). Some ovocytes undergo degeneration; mitotic divisions of gonia are not occurring, and there is a loosening of the normal stratification of the cortex. Some gonia are leaving the surface layer and drift toward the medulla of the gonad. At the same time the ovarian sacs decrease in size and the entire medulla (sac epithelia and ovarian rete) becomes more massive. In the case represented by Fig. 1 the presence of the melanophores in the hilum and along the sac epithelium indicates a renewed immigration of medullary blastema cells (of mesonephric origin). Melanophores are often seen in testes but never in normal

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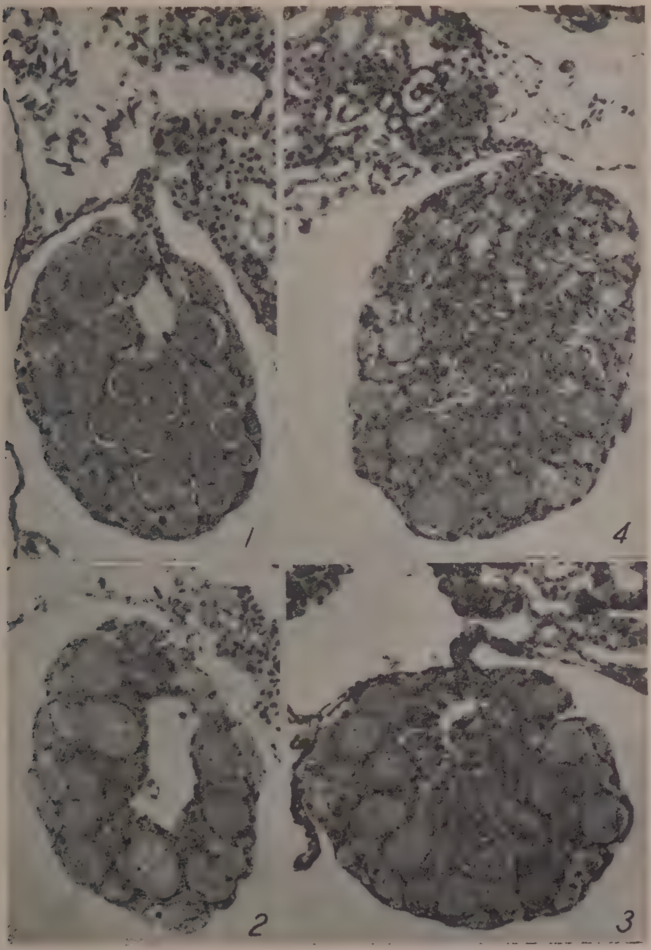

FIG. 1. Effect of cortisone on ovarian development; 1 mg/l, 25 days treatment. Reduction of the lumen of the ovarian sac; shifting of undifferentiated gonia toward the medulla; melanophores within the gonad. Ovarian character still predominant. ×167; larval stage 29.

FIG. 2-4. Effect of cortisone on ovarian development; 1 mg/l, 46 days treatment. Cross-sections through three sex glands illustrating successive steps in the transformation of ovaries into testes. The degenerative reduction of the cortex is followed by massive medullary proliferation. While the cavities of the ovarian sacs shrink, medullary cords grow peripherally and absorb the remaining undifferentiated gonia, which thus become spermatogonia. In Fig. 4 one notices the formation of seminal tubules and of an efferent ductule. All Figs. ×167; larval stages 30-32.

ovaries of this species; in the present case they constitute unequivocal evidence that sex reversal has become initiated.

The remaining 17 specimens were preserved during metamorphosis (stage 30) after 46 days of treatment. In this group normal or near normal ovaries are found no longer; it consists of 8 typical males and 9 hermaphrodites. The latter have ovaries in various stages of testicular transformation, up to near-completion of the process (Fig. 2 to 4). Since 171 controls consisted of 99 females and 72 males, and exhibited no tendency toward intersexuality, it is evident that sex reversal in the experimental lot was induced by the cortisone. Fig. 2 to 4 show how the degenerative processes in the ovarian cortex are immediately followed by thickening of the walls of the ovarian sacs, proliferation of numerous medullary cords, and differentiation of seminal tubules. The ovarian sacs become gradually reduced to seminal and reticular tubules of the testis.

The cortisone experiments are being continued, in order to obtain more information about the range of dosages, that will result in sex reversal; however, the fact that this substance like the other tested androstanes interferes with normal ovarian development and eventually causes sex reversal in females of *Rana sylvatica,* is now safely established. This result is somewhat surprising because cortisone has not, so far, been known to have androgenic functions. As a matter of fact, the results of our experiment should neither be considered as a demonstration of an androgenic character. This term should rather be restricted to designate the stimulation of male secondary sex characters. In our experiment cortisone evidently acts entirely by interfering with normal ovarian development. The development of the gonadal cortex becomes inhibited, and later the differentiated ovocytes are caused to degenerate. The transformations that lead to the development of the testicular structure probably are not induced directly by the cortisone. Since constitutionally the female larvae are bisexual, with the female determiners epistatic over the male factors, the inhibition or destruction of the ovarian cortex automatically creates the possibility for a compensatory, medullary-testicular development(3). The action of cortisone on the sex glands is, therefore, best characterized as antagonistic to the development of an ovarial cortex or, shortly, as *anti-ovariogenic.*

Summary. Cortisone, like 3 other tested alkyl substituted androstanes, causes the transformation of ovaries of frog larvae into testes. Its action is initially anti-ovariogenic, and sex reversal follows by a compensatory development of the medullary rudiment. The dosages that produce sex reversal are high in comparison with the threshold values of testosterone and methyltestosterone.

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Received October 30, 1950. P.S.E.B.M., 1950, v75.

Antigenic Similarity of Five Human Strains of Pleuropneumonia-like Organisms. (18318)

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Serologic studies of the pleuropneumonia-like organisms (hereafter referred to as PPLO) have been hampered by the difficulties encountered in isolating and maintaining strains. Cultural and morphologic characteristics of human strains have been studied, but relatively few observations employing serologic methods have been recorded. Warren and Sabin(1) compared one human strain with 5 mouse and 2 rat strains and found no serologic cross reactivity, confirming previous findings that strains from different animal species are antigenically diverse(2,3).

The PPLO have attracted attention as possible etiologic agents in non-gonococcal urethritis. Further information concerning this possible relationship may be gained by the application of serologic methods. Therefore, available strains were studied serologically with rabbit as well as with human sera. The preliminary phases of this study are reported herewith.

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Methods and materials. Six strains of PPLO from the male and female human genital tracts were included in this study and will be designated as follows: 225† (female), L-50† (female), Douglas† (female), Campo‡ (male), N3‡ (female) and J-"L"§ (male). Each of these strains conformed to the morphologic and cultural characteristics of the group. Cultures were maintained by weekly transfers to the cystine trypticase semi-solid medium previously described from this laboratory(4). This medium was employed in broth form (*i.e.* without agar) in preparing antigens for injecting rabbits and for testing antisera. Approximately 15 transfers at 4- to 6-day intervals were made into the broth in order to obtain a smooth homogeneous growth. For the final mass production of antigen, 15 ml of a 4-day broth culture were inoculated into 1 liter of the same medium. After 4 to 6 days incubation at 35°C, the culture was centrifuged at 4200 rpm for one hour. The resultant sediment was then washed in sterile physiologic saline 4 times at 4200 rpm for one hour and resuspended in sterile saline to match the opacity of a No. 3 MacFarland nephelometer tube. Those suspensions used for testing antisera were preserved with 1:5000 aqueous merthiolate and stored at 4°C. Approximately 30 to 50 ml of test antigen could be obtained from each liter of broth culture. Uninoculated broth also produced a sediment which, when diluted to match the No. 3 nephelometer tube, yielded about 10 to 20 ml from each liter.

Antisera were produced by progressive daily intravenous injections into rabbits of 1, 2, 3 and 4 ml of viable, *i.e.,* unpreserved antigen. Five days after the last injection (9th day), a trial bleeding was performed, after which the rabbits were given an additional injection of 4 ml, then bled from the heart on the 12th day. Control rabbits were injected similarly with the suspended sediment from the uninoculated broth. For the *agglutination test,* sera were inactivated at 56°C for 20 minutes and serial dilutions made in physiologic saline. An equal quantity (0.5 ml) of antigen was added to each tube. After thorough mixing, the tubes were incubated at 52°C for 18 hours, then centrifuged at 800 rpm for 5 minutes. Readings for degree of agglutination were made with the aid of a microscope mirror while the tubes were gently shaken. Complete agglutination was recorded when a floccular sediment appeared in a clear supernate. Tests showing a finely granular sediment were considered negative. Serum controls included sera from normal rabbits as well as from those injected with suspended sediment from the uninoculated medium. Saline-antigen mixtures and suspended sediment from uninoculated medium were used as antigen controls.

Absorption and cross-absorption studies were performed with the lyophilized antigens of the Douglas and Campo strains of PPLO, and their corresponding antisera. The dried powder from 1 ml of lyophilized antigen was resuspended in 1 ml of physiologic saline, the suspension centrifuged at 3200 rpm for 15 minutes, the supernate discarded and 1 ml of antiserum (diluted 1:5) added. The antigen-antiserum suspensions were well mixed and incubated at 52°C for 1 hour then centrifuged at 3200 rpm for 30 minutes. The supernates were diluted serially and the agglutination titers were determined by the same procedure used for the unabsorbed serum.

Results. Pre-immunization sera did not agglutinate 5 of the 6 strains of PPLO. Similarly, 12 other sera from normal rabbits and antisera for various *Salmonella* and *Shigella* strains failed to agglutinate suspensions of these 5 strains. The sixth strain, L-50, was agglutinated by both normal human and normal rabbit sera, thus was considered unsatisfactory for serologic study.

Antisera for all six strains agglutinated the homologous antigens in titers varying from 1:320 to 1:1280. Marked cross reactivity

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† Obtained from Lt. C. D. Graber, Third Army Area Medical Laboratory.

‡ Obtained from Dr. J. Warren, Army Medical Dept. Research and Graduate School.

§ Obtained from Dr. L. Dienes, Mass. General Hospital, Boston, Mass.

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TABLE I. Agglutination Titers of Antisera with Homologous and Heterologous Strains of Human Pleuropneumonia-like Organisms.

	Antigens							
Sera	225	L-50	Douglas	Campo	N3	J-"L"	Medium control*	Saline-antigen control
225	1:1280	1:320	1:640	1:1280	1:1280	1:320	0	0
L-50	1:1280	1:1280	1:640	1:640	1:1280	1:320	0	0
Douglas	1:1280	1:1280	1:1280	1:1280	1:640	1:640	0	0
Campo	1:1280	1:640	1:640	1:1280	1:1280	1:1280	0	0
N3	1:1280	1:640	1:1280	1:320	1:1280	1:640	0	0
J-"L"	1:1280	1:1280	1:1280	1:320	1:1280	1:1280	0	0
Control†	0	0	0	0	0	0	0	0
Normal rabbit	0	1:320	0	0	0	0	0	0

* Resuspended sediment from uninoculated medium.

† Serum from rabbit which was injected with the resuspended sediment of the uninoculated medium.

occurred between the strains, as can be seen in Table I. The homologous and heterologous sera agglutinated at the same dilutions, or differed in titer by only one or two dilutions. This would suggest that the individual organisms were antigenically similar. A recently isolated strain of *Salmonella typhimurium* grown in the same medium used for the PPLO, was not agglutinated by any of the antisera for PPLO.

Antisera from rabbits injected with the Douglas and Campo strains were tested after absorption with the Douglas and Campo antigens. The antibodies for the Douglas, Campo, N3 and J-"L" strains were removed completely from these two antisera by absorbing with either the Douglas or Campo strains. (Insufficient antigen from strain 225 was available at the time of these tests.) The results of the cross-absorption tests further indicate the antigenic similarity of the human strains of PPLO studied.

Sera obtained from 18 cases of non-gonococcal urethritis failed to agglutinate the five test strains, although PPLO had been isolated from only 2 of these patients. Thirty samples of human sera submitted for routine serological tests also failed to agglutinate any of the 5 human strains. These latter results are contrasted with the complement-fixation studies of Beveridge, Campbell and Lind(5) who found that a high percentage of normal human sera reacted positively with human strain antigens.

The time consumed in preparing antigens prompted an investigation into the possibility of using reconstituted suspensions of pleuropneumonia-like organisms which had been lyophilized and stored at 4°C for 3 months. The results with the lyophilized antigens were the same as those obtained with freshly prepared antigens.

Summary. 1. Suspensions of PPLO suitable for rabbit immunization and for use in agglutination tests were produced in relatively large quantities from cystine trypticase broth. 2. Five human strains of PPLO were closely related antigenically, as measured by agglutination titers with homologous and heterologous rabbit sera. These strains failed to agglutinate in the presence of normal rabbit and human sera. A sixth strain was agglutinated in all normal human and rabbit sera. 3. Further evidence was obtained for the antigenic homogeneity of the human PPLO studied by cross-absorbing 2 antisera and 2 antigens. After such absorption no antibody activity remained for any of the 4 strains tested. 4. Sera from 18 cases of non-gonococcal urethritis failed to agglutinate suspensions of 5 human strains of PPLO. 5. Stable suspensions were produced from antigens lyophilized for at least 3 months.

Received October 31, 1950. P.S.E.B.M., 1950, v75.

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Effects of Vitamin B_{12} in Multiple Sclerosis.* (18319)

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In view of the improvement of neurologic disease in patients with pernicious anemia following treatment with vit. B_{12} the following study was undertaken(1-3). During this period Lereboullet, Pluvinage and Coty(4) reported that there were satisfactory results observed in 7 patients with multiple sclerosis treated with vit. B_{12} in doses no greater than 30 μg every other day. Schumacher(5), on the other hand, observed no beneficial effects after 6 months of treatment with 30 μg of vit. B_{12} daily.

Material and methods. A group of 4 persons with multiple sclerosis was selected. Their ages were 32, 37, 39 and 42 years, and the duration of their disease was 11, 16, 11 and 7 years respectively. They had all been patients at the hospital for many years and adequate baseline records of their neurologic status, as well as responses to previous therapy, were available. Before treatment each patient had a complete hematologic survey consisting of a hemoglobin estimation, erythrocyte and leukocyte counts, reticulocyte studies, bone marrow examination, and a reevaluation of the neurologic status. Each case received 1050 μg of crystalline vit. B_{12}, intramuscularly, daily. Reticulocyte counts were done each day for 2 weeks. Complete blood studies including bone marrow surveys were made every 4 to 5 weeks. Three of the patients were treated for 5 months, the remaining one for 4 months. During this period frequent observations on the neurologic status were made by various examiners. One other patient, a 46-year-old male, with amyotrophic lateral sclerosis of 3 years' duration, was treated with 350 μg of vit. B_{12} intramuscularly, daily for 5 months.

Results. There were no untoward systemic reactions following injections of these large doses of crystalline vit. B_{12}. Occasional local pain was reported by some of the patients, but this was transient. No clinical improvement in neurologic status was observed in any of the 4 patients with multiple sclerosis, or the single patient with amyotrophic lateral sclerosis. In one patient there were signs of rapid deterioration during the course of treatment which was not attributed to the drug. No changes were noted in any of the peripheral blood or bone marrow examinations. An additional patient, 29 years old, with multiple sclerosis of one month's duration, was given 1050 μg of crystalline vit. B_{12} intramuscularly, daily, for 3 weeks. There were no signs of improvement and the medication was discontinued.

Summary. Five patients with multiple sclerosis and one patient with amyotrophic lateral sclerosis failed to respond to massive daily intramuscular injections of crystalline vit. B_{12}.

Received October 30, 1950. P.S.E.B.M., 1950, v75.

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* Crystalline vit. B_{12} was supplied by Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y.

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Production of a Nutritional Deficiency in Mice Fed a Low Fat Diet. (18320)

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Variations in dietary composition with regard to fat, carbohydrate, or protein markedly influence an animal's requirements for certain of the vitamins. It has been shown, for example, that the vit. B_{12} requirement for growth of the mouse is influenced by the fat and protein levels in the diet(1). Other factors may influence dietary vitamin requirements. Among these are stresses, such as the feeding of thyroid active materials, or the administration of intestinal bacteriostatic agents that suppress the synthesis of essential nutrients by the intestinal flora. During the course of investigations involving possible interrelationships between dietary composition, stress factors, and the administration of intestinal bacteriostatic agents it was found that the addition of succinylsulfathiazole (SST) to a diet low in fat but otherwise complete with regard to all other known essential nutrients caused a growth retardation in mice.

This study was undertaken to investigate the nature of this SST-induced deficiency.

TABLE I. Composition of Basal Diet.

Corn oil*	0.5
Casein†	20
Glucose‡	20
White dextrin	53.5
Salt mixture(2)	4
Cellu flour	2
Each 100 g of diet supplemented with:	
Vit. A	900 U.S.P. units
Vit. D	180 U.S.P. units
α-tocopherol	4 mg
2-methyl-1,4-naphthoquinone diacetate	1 ’’
Thiamine hydrochloride	0.8 ’’
Riboflavin	1.6 ’’
Pyridoxine hydrochloride	0.8 ’’
Niacin	4.0 ’’
Ca pantothenate	4.4 ’’
Para-aminobenzoic acid	4.0 ’’
Choline chloride	200.0 ’’
Inositol	21 ’’
Folic acid	0.2 ’’
Biotin	0.02’’

* Mazola, Corn Products Refining Co., Argo, Ill.

† Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, O.

‡ Cerelose, Corn Products Refining Co., Argo, Ill.

Jones and Foster(2).

Each mouse received 0.1 μg of vit. B_{12} per day by weekly intraper. inj.

Experimental and results. The basal ration employed in these studies had the composition shown in Table I. Although this diet is low in fat (0.5%), sufficient vitamin B_{12} to assure optimal growth was given(1). All substitutions in this diet were made at the expense of equivalent weights of white dextrin. The various diets were fed *ad libitum* to groups of male weanling mice (Sharp and Dohme, Swiss Webster strain) for 2-week periods.

Good growth was obtained with the basal diet. However, when 2% SST was incorporated in the diet a marked retardation in growth rate resulted. Doubling the dietary levels of all the known vitamins had no effect on the SST-induced growth retardation. Growth rates were improved, however, when fat or certain vegetable materials such as defatted cottonseed meal, rolled oats, or dried grass were added to the basal diet. All liver fractions that were tested showed a marked toxicity. The deleterious effect of liver could be prevented by the addition to the diet of fat or cottonseed meal. The results of this portion of the study are shown in Table II.

A second study was made to determine the effect of increasing the SST level in the diet from 2% to 4%. The effect of varying levels of cottonseed meal and of fat also was determined with the higher SST level. The results, shown in Table III, indicate that diets containing 4% of SST caused a greater growth retardation than did those containing 2% of SST. Both cottonseed meal and fat were effective in restoring growth rates to the control values but higher levels of cottonseed meal were necessary when the diet contained

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1. Bosshardt, D. K., Paul, Winifred J., and Barnes, R. H., *J. Nutrition,* 1950, v40, 595.

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TABLE II. Effect of Succinylsulfathiazole (SST), Fat, and Natural Products on Growth of Mice.

Dietary supplement	No. of mice	Avg gain in body wt, 14 day period (g)
None	64	8.5 (59)
2% SST	64	5.0 (57)
’’ ’’ + 3% partially defatted cottonseed meal*	56	7.4 (52)
’’ ’’ + 3% petroleum ether extracted Proflo	8	7.3 (8)
’’ ’’ + 3% Cerophyll powder†	8	6.2 (6)
’’ ’’ + 3% rolled oats	8	7.5 (7)
’’ ’’ + 3% distiller’s dried solubles	8	2.3 (6)
’’ ’’ + 3% Bacto yeast extract	8	3.9 (2)
’’ ’’ + 3% wheat germ	8	4.5 (8)
’’ ’’ + 3% dried yeast‡	8	4.7 (8)
’’ ’’ + 3% fish solubles	8	4.5 (4)
’’ ’’ + 3% dried beef muscle	8	3.6 (8)
’’ ’’ + 3% olive oil	8	6.5 (7)
’’ ’’ + 25% olive oil	8	7.1 (8)
’’ ’’ + 3% lard	8	5.4 (7)
’’ ’’ + 25% lard	8	8.5 (8)
’’ ’’ + 3% corn oil§	8	6.7 (7)
’’ ’’ + 25% corn oil	8	8.0 (8)
’’ ’’ + 3% butterfat	8	5.7 (8)
’’ ’’ + 25% butterfat	8	7.6 (8)
’’ ’’ + 3% cottonseed oil	8	5.3 (8)
’’ ’’ + 25% cottonseed oil	8	8.4 (7)
’’ ’’ + 25% hydrogenated cottonseed oil‖	8	9.3 (8)
’’ ’’ + 25% wheat germ oil	8	9.5 (8)
’’ ’’ + 25% peanut oil	8	8.6 (8)
’’ ’’ + 3% lyophilized fresh liver	8	1.3 (6)
’’ ’’ + 3% Wilson’s liver fraction L	8	—1.1 (4)
’’ ’’ + 3% Wilson’s liver fraction S	8	—0.5 (5)
’’ ’’ + 3% defatted, desiccated liver¶	8	2.9 (3)
’’ ’’ + 3% defatted, desiccated liver¶ + 25% hydrogenated cottonseed oil‖	8	10.3 (8)
’’ ’’ + 3% Wilson’s 1:20 liver powder	8	0.5 (2)
’’ ’’ + 1% Wilson’s 1:20 liver powder	8	1.8 (6)
’’ ’’ + 1% Wilson’s 1:20 liver + 3% cottonseed meal*	8	7.9 (8)

The figures in parentheses are the number of survivors and numbers on which average weight gains are based.

* Proflo, Traders Oil Mill Co., Fort Worth, Texas.
† Cerophyll Laboratories, Inc., Kansas City, Mo.
‡ Strain G, Anheuser Busch Co., St. Louis, Mo.
§ Mazola.
‖ Primex.
¶ VioBin.

4% of SST.

Discussion. It has long been recognized that fat has a beneficial effect on growth. Since the findings of Burr and Burr(3) were published, it has been recognized that one of the factors involved is the content of the "essential" unsaturated fatty acids in the dietary fat. White, Foy, and Cerecedo(4) noted that mice grew better when fed a diet containing 10% lard than when fed a fat-free diet. Furthermore, the mice fed the 10% lard diet did not develop the dermatitis typical of an essential fatty acid deficiency that was noted with the mice fed a fat-free diet. Although the dermatitis appeared on the 30-40th day, a growth difference was noted from the start of the experiment. This would suggest a secondary deficiency; it has been stated by Burr(5) with reference to his work with rats, "If the rats do not grow rapidly at first some other deficiency should be suspected". It has been shown by Deuel and coworkers(6) that fat *per se* has a beneficial effect on the growth of rats. This effect was not affected by the incorporation of methyl linolate in the basal fat-free diet.

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3. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, v82, 345; 1930, v86, 587.

4. White, E. A., Foy, J. R., and Cerecedo, L. R., Proc. Soc. Exp. Biol. and Med., 1943, v54, 301.

5. Burr, G. O., *Fed. Proc.*, 1942, v1, 224.

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TABLE III. Effect of Succinylsulfathiazole (SST), and Cottonseed Meal on Growth of Mice.

Supplement	No. of mice	Avg gain in body wt, 13-day period (g)
None	72	7.6 ± .35* (70)
2% SST	32	5.7 ± .54 (29)
" " + 3% cottonseed meal	32	8.0 ± .50 (29)
4% SST	40	4.1 ± .47 (39)
" " + 3% cottonseed meal	8	6.9 ± .76 (7)
" " + 4% cottonseed meal	8	7.1 ± .97 (8)
" " + 5% cottonseed meal	40	7.8 ± .38 (39)
" " + 10% cottonseed meal	8	8.3 ± .91 (7)
" " + 20% Primex	8	7.2 ± .70 (7)

The figures in parentheses are the numbers of survivors and numbers on which average weight gains are based.

$$* \text{ S.E.} = \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}.$$

The results obtained in this study show that the addition of SST to an apparently complete diet caused a marked growth retardation. Fat and several natural products of vegetable origin counteracted this effect. It seems unlikely that the fat effect is due to the intake of linoleic acid. The basal diet, containing 0.5% corn oil, contains 200 mg of linoleic acid per 100 g of diet. Regardless of the fat source, better growth was obtained with diets containing 25% of fat than with those containing 3% of fat. However, as shown in Table IV, 3% of fats such as cottonseed oil, peanut oil, corn oil, or wheat germ oil contributed as much or more linoleic acid to the diet as did 25% of butterfat, lard or olive oil. It would appear from these data that the fat effect is not due to the essential fatty acids present but rather to the fact that fat *per se* is an essential nutrient.

If lipids are essential nutrients for animal growth they must either be supplied in the diet or synthesized by the animal from other foodstuffs. It is known that animals are able to synthesize lipids from intermediary metabolites of carbohydrate and certain amino acids. If it can be assumed that the sole role of SST is that of an intestinal bacteriostatic agent the results obtained in this study would suggest that a factor essential for lipid synthesis by the animal is formed by the intestinal flora. The results obtained with cottonseed, oats, and grass would indicate that these materials contain this factor.

TABLE IV. Dietary Levels of Linoleic Acid.*

	Fat level in diet*	
Supplement	3% mg/100 g diet	25% mg/100 g diet
Butterfat	120	1000
Lard	183	1525
Olive oil	210	1750
Cottonseed oil	1500	12500
Peanut oil	750	6250
Corn oil	1200	10000
Wheat germ oil	1470	12250

* Linoleic acid values are those reported by Hilditch(7).

† In addition each diet contained 200 mg linoleic acid per 100 g diet contributed by the 0.5% corn oil in the basal diet.

The inhibitory effects of the liver preparations on the growth of mice fed low fat diets containing SST cannot be explained at present. Since the liver effect can be counteracted by the addition to the diet of fat or cottonseed meal it is possible that liver contains an antagonist or an anti-metabolite for the postulated factor or factors. If such is the case it is possible that some of the materials shown to be inactive (Table II) may have contained the factor, or factors, but that a beneficial effect was masked by the presence of an antagonist.

It appears improbable that the postulated factor/s, is the vitamin B_{13} of Novak, Hauge, and Carrick(8) although the basal diets were quite similar. Several sources of vitamin B_{13}

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8. Novak, A. F., Hauge, S. M., and Carrick, C. W., *Poultry Sci.*, 1947, v26, 604.

such as fish solubles, liver, and distillers' dried solubles were found to be inactive. However, it has been impossible to confirm the results of Novak *et al.* using mice. Likewise it is not known whether or not the postulated factor/s, is identical with that proposed by Ruegamer(9) or by Zucker and Zucker(10).

Summary. The addition of succinylsulfathiazole to low fat diets caused a growth retardation in mice although adequate quantities of all known accessory growth factors were fed. Normal growth resulted when fat, cottonseed meal, or rolled oats were added to the basal diet containing succinylsulfathiazole.

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Received November 6, 1950. P.S.E.B.M., 1950, v75.

Thromboplastic Potency Changes. A Result of Bacterial Action. (18321)

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Recent work in this laboratory has shown a significant factor in the potency deterioration of tissue extracts currently used as sources of thromboplastin to be associated with the activity of a bacterial contaminant, *B. subtilis*, in the thromboplastic suspensions.

It is the purpose of this paper to describe this effect and some properties of thromboplastic suspensions so altered.

Materials and methods. Rabbit brain and rabbit lung were used as sources of thromboplastic powder. Details of preparation of thromboplastic powders and their extraction to yield the final suspensions used, have been described elsewhere(1). Diluent for the thromboplastic suspension was 1:1 NaCl-$CaCl_2$ mixture (see below). Normal human oxalated plasma was employed (1 vol. of 0.1 M sodium oxalate to 9 vol. of freshly drawn whole blood). Ten per cent prothrombin plasma was obtained by diluting 100% prothrombin plasma with prothrombin-free plasma critically adsorbed(2) with $Ca_3(PO_4)_2$. All plasma was kept in an ice-water bath when not in use. Saline 0.85% NaCl and calcium 0.025 M $CaCl_2$ were used. Clotting time determinations: Our modification (1) of the Quick one-stage method was used, employing chemically clean glassware throughout. *B. subtilis* was cultured from a thromboplastic suspension which had undergone alteration upon incubation (see below). Routine bacteriological technic was employed. Difco brain-heart infusion with 2% agar was used as the plating medium. During the course of a day's routine clinical prothrombin determinations it was observed that the potency of the thromboplastic suspension decreased sharply during the period of the determinations. Observations made during a controlled study of the nature of the change in this batch of thromboplastic material was subsequently found to apply to all batches of brain and lung powders investigated.

Results. Brain thromboplastic suspension was incubated at 37.5°C for a period of 9 hours in a test tube open to air. During this time, oxalated plasma was kept in an ice-water bath and 0.1 cc portions removed as needed. Little change in clotting time occurred for the first 5 hours (Fig. 1). Between 5½ and 6 hours, however, there was a sudden, rapid prolongation of clotting time. The plasma, when tested at this time with a fresh thromboplastic suspension clotted in 12.4 seconds. The sudden prolongation in clotting time was therefore attributed to the loss in potency of the incubated brain thromboplastic suspension. A similar loss in potency was shown to occur with lung thromboplastic sus-

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1. Gollub, S., Kaplan, F. E.. and Meranze, D. R., *Am. J. Physiol.*, 1950, v162, 293.

2. Gollub, S., Kaplan, F. E., Meranze, D. R., and Tuft, H., *Am. J. Clin. Path.*, 1949, v19, 1021.

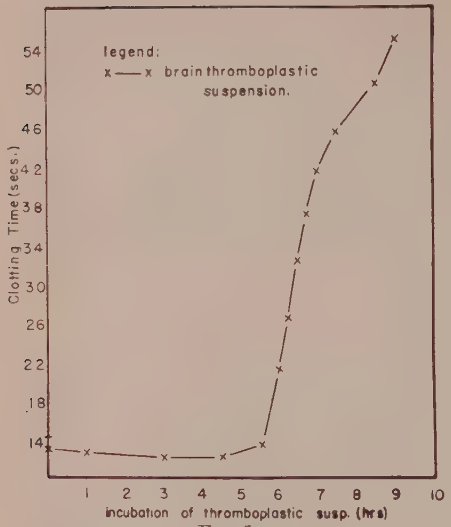

FIG. 1.

Characteristic curve showing suddenly prolonged clotting times obtained with normal human plasma when brain thromboplastic suspension is kept in open test tube at 37.5°C several hours.

pension in repeated experiments. The time of the "break" in the curve varied from 5 to 12 hours depending upon the sample of powder employed in making the thromboplastic extract. Such thromboplastic suspensions which have undergone apparent sudden loss of potency on incubation will hereafter be designated △ thromboplastic suspensions.

Prevention of deterioration. An attempt was made to shorten the prolonged interval before the sudden loss in potency by increasing the temperature of incubation 10°C. The unexpected finding that incubation at 47.5°C prevented the sudden loss of potency suggested that the observed effect might be the result of bacterial contamination. We therefore studied the effects of temperature and bactericides, simultaneously with bacteriological studies. It was found that the prevention of the sudden loss in potency of thromboplastic suspension could also be accomplished with phenol in final concentration of 0.25% as well as with "Mercurophen"* in final concentration of 0.02% (Fig. 2). Incubation at 47.5°C with subsequent incubation at 37.5°C caused a delay in the "break" in the curves. Concentrations of phenol and mercurophen lower than those given above also caused delay. Transferring thromboplastic suspension to 47.5°C after incubation at 37.5°C prevented or very greatly delayed the "break." However, once the "break" in the curve had started at 37.5°C, incubation at 47.5°C neither hindered nor stopped it.

During the course of incubation of brain thromboplastic suspension, clotting times with 100% prothrombin plasma were recorded. Simultaneously, 0.1 cc portions of brain thromboplastic suspension were removed and dilutions of 10^{-5}, 10^{-6} and 10^{-7} or 10^{-6}, 10^{-7} and 10^{-8} were made. Agar plate counts of bacterial colonies were made after 24 hours incubation at 37.5°C. The average count of 3 dilutions was recorded. The variation of the counts was somewhat large but the counts were all of the same order of magnitude (Fig. 3). There is a coincidence in the time of the sudden loss of potency of thrombo-

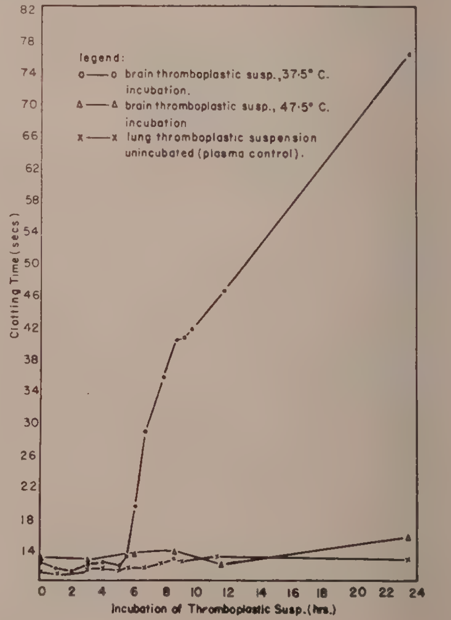

FIG. 2.

Showing that the sudden loss of potency can be prevented or greatly delayed by incubation of thromboplastic suspensions at 47.5°C. Phenol and Mercurophen are also effective.

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* Sodium - oxy-mercury - ortho - nitro-phenolate. Sharp and Dohme.

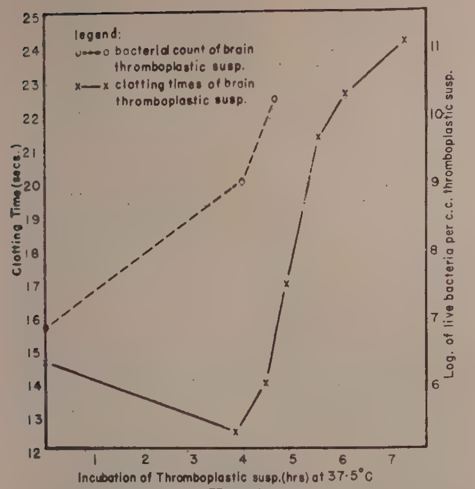

FIG. 3.

Showing the relationship between growth of organism (*B. subtilis*) in the thromboplastic suspensions and the sudden loss of potency.

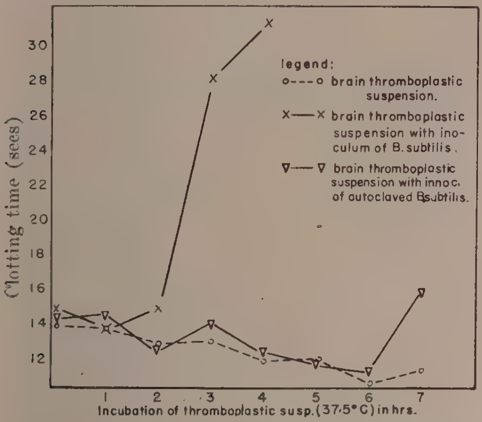

FIG. 4.

Showing that the sudden loss of potency of thromboplastic suspensions occurs sooner when *B. subtilis* is added initially to the suspension.

plastic suspension and the sudden increase in the colony count (number of organisms in the thromboplastic suspension). The organisms of the colonies which plated out of the △ suspension were uniformly found to resemble *B. subtilis*. This was confirmed by additional bacteriologic examinations.

To speed the occurrence of the "break" in the clotting time curve, it seemed advisable to add initially to the thromboplastic suspension, a concentrated suspension of an active form of the isolated *B. subtilis*. Accordingly, brain thromboplastic suspension was prepared and divided into 3 portions, one receiving no treatment, another receiving 0.3 cc of inoculum per 10 cc thromboplastic suspension, and a third receiving 0.3 cc of autoclaved inoculum per 10 cc of thromboplastic suspension. These 3 samples were incubated at 37.5°C and clotting times of each with 100% prothrombin plasma were determined. The data are given in Fig. 4. The inoculum of living organisms drastically reduced the time for the "break" in the curve. The clotting times obtained with the sample receiving autoclaved inoculum were indistinguishable (in the interval investigated) from those obtained with the untreated portion. The reduction of time for the break in the curve is even more striking if one incubates the inoculated thromboplastic suspension in vessels which allow greater air-liquid interfaces (*e.g.* Erlenmeyer flasks). Thus the sudden loss of potency of these thromboplastic suspensions appeared to

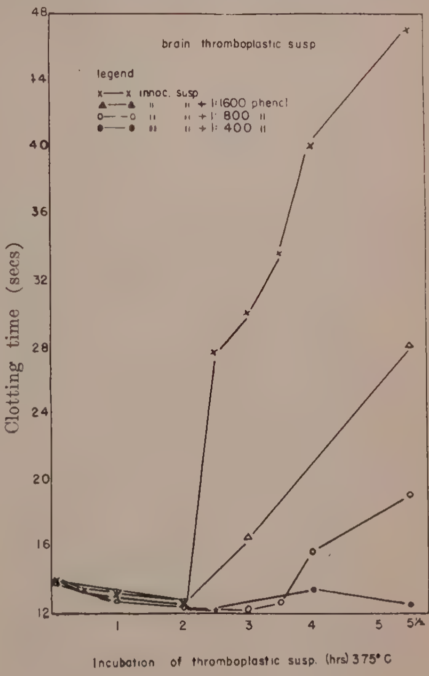

FIG. 5.

Showing the effect of various concentrations of phenol upon sudden loss of potency of preinoculated (*B. subtilis*) thromboplastic suspensions.

be associated with the vital activity of the organism.

Having thus reduced the interval before the sudden loss of potency it seemed of interest to determine the effects of phenol on such an inoculated suspension. Fig. 5 summarizes the data. Again it is seen that a final concentration of 1:400 (0.25%) phenol prevented the sudden loss of potency in the interval investigated. Lower concentrations of phenol were decreasingly effective.

Some aspects of the functioning of the △ thromboplastic suspensions. (1) Using unaltered (pre △) thromboplastic suspension, the clotting times of 100% and 10% prothrombin plasmas were determined at 5 minute intervals in the neighborhood of the "break" in the curve. The "break" in the curve was found to be simultaneous for both plasmas. (2) The clotting times of dilutions of △ suspensions were determined with 100% and 10% prothrombin plasmas. The clotting times for both 100% and 10% prothrombin plasma increased initially and progressively upon diluting the △ thromboplastins. (3) Prior to incubation, a portion of thromboplastic suspension was removed and refrigerated. After the incubated portion had undergone the sudden loss in potency (△ suspension) its clotting time with 100% prothrombin plasma was recorded. The refrigerated, unincubated portion of thromboplastin-$CaCl_2$ was then diluted until its clotting time with 100% prothrombin plasma was the same as that of the △ suspension. (The diluted, unincubated portion of thromboplastin is hereafter referred to as the dilution analogue.) In table I are shown the clotting times of △ suspensions of brain and lung and their dilution analogues on 100% and 10% prothrombin plasma. Though the 100% prothrombin clotting time for △ and dilution analogue suspensions were made to coincide, the 10% prothrombin clotting times of the △ suspensions are markedly longer than those obtained with their dilution analogues.

The results of this study have been found to hold true for lung thromboplastic suspensions as well.

Discussion. These data clearly demonstrate that the activity of *B. subtilis* caused a sudden loss in potency of brain and lung thromboplastic extracts. Preliminary experiments which indicate a separable enzyme-like material as the causative agent will be reported shortly. It is not yet possible to say whether the effect (apparent loss of potency) was due to elaboration of inhibitor, destruction of activator, or both. The data showing concomitant rise in clotting time with 100% and 10% prothrombin plasma can be interpreted as evidence against the destruction of activator. The reasoning is as follows: If activator were destroyed, the sudden rise in clotting time with 100% prothrombin should precede the sudden rise in clotting time with 10% prothrombin. This is true because the destruction of activator should result in a sub-optimal concentration of activator for 100% prothrombin plasma earlier than for 10% prothrombin plasma. Similarly, the clotting time data obtained with dilutions of thromboplastic suspension argue against the elaboration of a type of inhibitor previously described(2) since clotting times of 100% as well as 10% prothrombin increased steadily when tested with more dilute thromboplastic suspension. Had such an inhibitor been elaborated, one would have expected an initial decrease of 10% prothrombin clotting time upon thromboplastic dilution(2).

TABLE I. Longer 10% Prothrombin Clotting Time with △ Thromboplastin than with Diluted Uninoculated Thromboplastin of Same Potency on 100% Prothrombin.

	BTP		LTP	
	△ suspension	Dilution analogue	△ suspension	Dilution analogue
100% Pt.	31.0	30.3	18.7	17.8
10% Pt.	77.0	35.3	74.0	35.0

Conclusions. A resumé of results thus far indicates that a sudden loss of potency of thromboplastic suspension occurred upon open-air incubation at 37.5°C which could be prevented or significantly delayed (up to 24 hours at least) with 47.5°C incubation or with phenol or "Mercurophen." This sudden loss of potency was coincident with the log. growth phase of an organism isolated from the thromboplastic suspension and identified as *B. subtilis*. Inoculation of thromboplastic suspension with a physiologically active form of *B. subtilis* drastically reduced the interval before the abrupt prolongation of clotting time. This also could be prevented (up to 7 hours at 37.5°C at least) with 0.25% phenol. The fact that *B. subtilis* can alter the apparent thromboplastic potency of tissue suspensions seems to have practical value as well as theoretical promise. From the practical aspect, the utilization of thromboplastic suspension can be extended by simple addition of phenol in cases where the potency loss is due to bacterial action. From the theoretical aspect, it would appear that the △ suspensions have an altered activator-inhibitor content. Such suspensions are amenable to investigation leading to possible concentration, or separation of activator or inhibitor. It is apparent from these preliminary observations that the nature of the changes of the incubated thromboplastic suspensions are yet to be explained. It is clear, however, that the marked changes induced by this bacterium and controlled by simple conditions offer new methods of investigating the content and nature of thromboplastic materials without resorting to drastic physicochemical treatment.

Summary. Data have been presented which show that a bacterial contaminant identified as *B. subtilis* caused a sudden deterioration in potency of brain and lung thromboplastic suspensions, which could be prevented by various agents. Some aspects of the functioning of the altered thromboplastic suspensions are presented and their significance discussed.

We wish to thank Mr. David C. Schechter for technical assistance.

Received November 6, 1950. P.S.E.B.M., 1950, v75.

Effects of Thyroidectomy and of Thiouracil on Adrenal Weight and Ascorbic Acid. (18322)

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It has been demonstrated that thyroid substances induce an enlargement of the adrenal glands(1,2), whereas thyroidectomy, or the administration of goitrogenic substances, results in a decrease in adrenal size(2-4). These alterations in adrenal size are restricted to the cortex of the gland. Long(5) and Sayers and Sayers(6) have reviewed the recent wide use of adrenal ascorbic acid content as an index of adrenocortical activity. The changes in adrenal ascorbic acid following adrenal stimulation or depression are also confined to the cortex. The effects of thyroid material on adrenal size and ascorbic acid content have already been reported by Sure and Theis(7) and by Wallach and Reineke(8). The present investigation was undertaken to determine the effects of hypothyroidism on the adrenal weight and adrenal ascorbic acid concentration of the male rat. Since it has been shown by Gordon, Goldsmith, and Charipper(9) that thyroidectomy and thiouracil exert somewhat

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TABLE I. Effects of Hypothyroidism on Adrenal Weight and Ascorbic Acid Concentration After 1, 2, and 4 Weeks of Treatment.

Wk	Group	Body wt,* g	Gain or loss,† g	Adrenal wt, mg	Ascorbic acid µg/mg adrenal	Thyroid wt, mg
1	1	157 ± 3	+24 ± 3	18.5 ± 2.2	5.67 ± .15	26.8 ± .9
	2	159 ± 7	+27 ± 1	22.3 ± 1.3	5.20 ± .33	15.1 ± .7‡
	3	167 ± 8	+31 ± 4	23.9 ± 2.1	5.59 ± .10	16.8 ± .6
	4	183 ± 9	— 4 ± 3	23.6 ± 1.7	5.71 ± .17	—
	5	190 ± 17	— 3 ± 8	27.3 ± 1.2	5.80 ± .20	—
2	1	133 ± 4	+42 ± 1	17.4 ± 0.7	4.70 ± .13	37.5 ± 1.3
	2	135 ± 4	+44 ± 1	21.0 ± 0.6	4.76 ± .35	14.9 ± .7
	3	133 ± 9	+46 ± 4	24.1 ± 1.4	5.57 ± .19	15.5 ± 1.1
4	1	153 ± 8	+38 ± 3	15.8 ± 0.8	4.83 ± .14	73.7 ± 2.5
	2	199 ± 6	+61 ± 2	24.3 ± 1.3	4.33 ± .14	21.2 ± .7
	3	184 ± 7	+44 ± 3	27.2 ± 1.7	4.31 ± .22	17.0 ± 1.4
	4	253 ± 16	+33 ± 5	29.9 ± 2.2	3.67 ± .25	—
	5	212 ± 8	+17 ± 4	32.8 ± 1.8	4.74 ± .17	—
Controls§		133 ± 5	—	22.4 ± 0.8	4.63 ± .07‖	16.1 ± .7¶
		158 ± 7	—	24.0 ± 1.4		
		189 ± 4	—	27.1 ± 0.9		
		227 ± 3	—	28.9 ± 1.5		

All values are means ± standard error. Group 1, thiouracil; 2, thiouracil + thyroxin; 3, thyroxin; 4, thyroidectomy; 5, thyroidectomy + thyroxin. Five rats per group in each period.

* At sacrifice. † Final minus initial body weight. ‡ 4 animals. § 6 animals per group. ‖ Value for all controls (25). ¶ Value for 12 animals.

different effects on metabolism, both methods of inducing hypothyroidism were employed. In addition, an estimated physiological dose (8) of thyroxin was administered daily to animals of both hypothyroid groups to determine whether such thyroidal replacement would prevent the adrenal changes associated with the experimental treatment.

Materials and methods. Sixty-five young male rats of a modified Long-Evans strain were separated into 5 experimental groups. The animals in Group 1 received thiouracil mixed thoroughly in their powdered complete ration in a concentration of 0.2%. Group 2 received the same thiouracil-supplemented diet plus daily subcutaneous injections of 5 γ of d,l-thyroxin per 100 g body weight in 0.1 cc saline. Group 3 received only the thyroxin. The animals in Group 4 were surgically thyroidectomized under ether anesthesia. Group 5 was thyroidectomized and also received 5 γ of thyroxin per 100 g body weight per day beginning with the first day of operation. Groups 1 through 3 contained 15 animals each, which were sacrificed in groups of 5 after 1, 2, and 4 weeks of treatment. Groups 4 and 5 contained 10 animals each, which were sacrificed in groups of 5 at 1 and 4 weeks after the start of treatment. One control group of 3 animals received subcutaneous injections of 0.1 cc of saline per day for one week, and another group of 3 animals were sham-operated and sacrificed one week later. A group of 25 animals was used for determination of normal adrenal weight and ascorbic acid, and normal thyroid weight was ascertained in 12 of these animals.

All animals were sacrificed by exsanguination. The adrenals were dissected out, cleanly trimmed of adhering fat, and weighed to the nearest tenth of a milligram on a torsion balance. The thyroids, in Groups 1 through 3, were also removed and weighed. After weighing, the adrenals of each animal were extracted with portions of a 6% solution of trichloroacetic acid, and adrenal ascorbic acid was then determined by the method of Roe and Kuether(10) with the dinitrophenylhydrazine reagent prepared by the method of Lowry, Lopez, and Bessey(11).

Results. The data obtained after 1, 2, and 4 weeks of treatment are shown in Table I, with values for normal animals of the same

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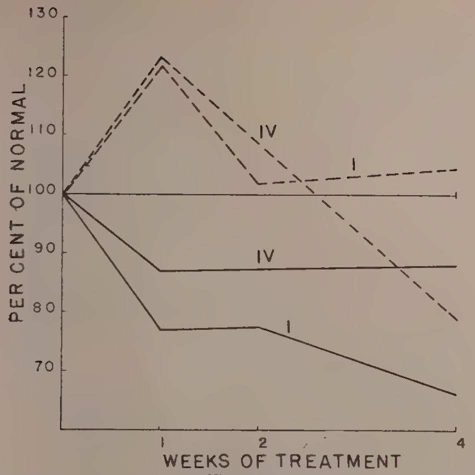

Fig. 1.

Adrenal ascorbic acid concentration (broken line), and adrenal weight (solid line), as % of normal after 1, 2, and 4 weeks of treatment. I, thiouracil group; IV, thyroidectomized group.

body weight.

The adrenal ascorbic acid concentrations and the thyroid weights of the controls showed no significant changes over the body weight range used and, therefore, the values for all control animals were pooled. On the other hand, the adrenal weights varied significantly with body weight in the normal animals, so that adrenal weights in each of the experimental groups are compared to those of normals of the same body weight range.

It can be seen from Fig. 1 that the adrenal atrophy was greater in the thiouracil-treated than in the thyroidectomized group; this same result was found by LeBlond and Hoff(3) and by Deane and Greep(2) with approximately the same concentration of thiouracil. Three sham-operated animals (not shown in the table) were found to have adrenal weight values 8% higher than normal after 1 week.

The data of Fig. 1 indicate that in Group 1 the increase in ascorbic acid concentration at the end of the first week was equivalent in magnitude to the decrease in adrenal weight; at 2 and 4 weeks the concentrations did not differ significantly from normal. In Group 4, however, the increase in ascorbic acid concentration at 1 week and the fall below normal at 4 weeks exceeded in magnitude the adrenal weight changes observed at the same times.

Fig. 2 shows that the daily dose of thyroxin administered to thiouracil-treated animals overcame almost completely the effects of the goitrogen. At 4 weeks, however, there was still a slight thyroid enlargement. The decreased food intake of thyroidectomized or thiouracil-treated animals has been reported (9,12). It was noted in this experiment that the animals receiving thyroxin in addition to thiouracil had a greater food consumption than those receiving only the goitrogen. Thus the animals in Group 2 consumed a greater absolute amount of the goitrogenic compound per day than did the animals in Group 1.

In Group 5 (Fig. 2) the adrenal weights did not differ from normal at any of the experimental periods, and the ascorbic acid concentration did not fall below normal at 4 weeks as in Group 4.

The level of thyroidal stimulation employed in Group 3 (Fig. 2) gave results approximating those obtained by Wallach and Reineke (8) who used a greater dosage. The ascorbic acid concentration in the adrenals of 3 animals receiving 0.1 cc of saline per day for 1 week

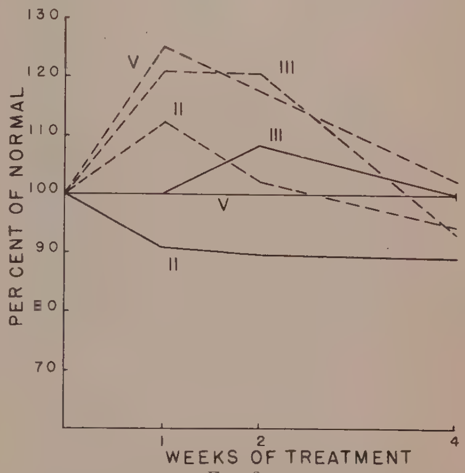

Fig. 2.

Adrenal ascorbic acid concentration (broken line), and adrenal weight (solid line), as % of normal after 1, 2, and 4 weeks of treatment. II, thiouracil + thyroxin group; III, thyroxin group; V, thyroidectomized + thyroxin group.

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(not shown in the Table) was not found to be significantly different from normal.

Discussion. The initial high ascorbic acid concentration seen after 1 week of treatment in the thiouracil-fed group, equivalent in magnitude to the atrophy of the adrenals, may reflect an increased storage by the adrenal cortex which, at this time, retains the normal total content of the vitamin. The normal ascorbic acid values noted in the later periods, in the atrophied glands, are interpreted as indicating an adjusted rate of synthesis, or mobilization, of the vitamin, with each unit mass of tissue secreting at a normal rate but the total adrenocortical output much reduced.

In the thyroidectomized rats and the thyroidectomized animals given thyroxin the initial increase, by the first week, in adrenal ascorbic acid probably represents a later phase of the response to the stress of the operation. The importance of the role of stress at the first week following the operation is suggested by the adrenal hypertrophy of the sham-operated animals, and by a previous observation (unpublished) that there is a sharp drop in adrenal ascorbic acid within 48 hours after the removal of the thyroids. The final decrease (at 4 weeks) in adrenal ascorbic acid concentration in the thyroidectomized animals exceeds the atrophy of the adrenals, and is believed to demonstrate the eventually greater effectiveness of thyroidectomy than of thiouracil in repression of cortical function. These results indicate a decreased rate of synthesis, or mobilization, of the vitamin and a more greatly reduced adrenocortical output, probably adequate, however, to handle the lowered metabolic needs of this type of animal(6).

The data obtained with thyroidal replacement in both hypothyroid groups support the view that the observed adrenal changes are due actually to changes in the level of thyroidal function. Experiments are required to determine whether the adrenal changes in hypothyroidism result from a decrease in the output of adrenocorticotrophic hormone (perhaps because of the accelerated production and release of thyrotrophic hormone by the pituitary) or whether the adrenal also responds directly to the lowered levels of thyroid hormone in the blood. In prolonged stress Pinchot, *et al.*(13) have questioned the reliability of ascorbic acid alone as an index of cortical activity. The results reported here demonstrate that the wide use of adrenal ascorbic acid concentration as an index of adrenocortical function is valid only if the changes are interpreted in terms of alteration in adrenal size.

Summary. The administration of thiouracil to the male rat results in a pronounced atrophy of the adrenal glands and an initial increase in ascorbic acid concentration in the adrenals followed by a return to normal values. Surgical thyroidectomy produces a less pronounced adrenal atrophy but a greater adrenal inhibition as revealed by a final decrease in adrenal ascorbic acid concentration. The effects of both types of induced hypothyroidism on the adrenals may be prevented by the administration of thyroxin. It is concluded that adrenal ascorbic acid concentrations are reliable indices of adrenocortical activity only if interpreted in terms of change in adrenal size.

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Received November 6, 1950. P.S.E.B.M., 1950, v75.

Immunization of Mice with Ultraviolet Killed Tuberculosis Vaccines.* (18323)

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There is increasing evidence that ultraviolet inactivated vaccines can be prepared for a number of viruses which are significantly more antigenic than heat or chemically inactivated control vaccines. We have prepared an effective irradiated rabies vaccine which is consistently more immunogenic than control phenolized vaccines(1) and which is widely used at present for both human and canine vaccination. We have also prepared an irradiated poliomyelitis vaccine which produces significant immunity to intracerebral challenge inoculation in vaccinated mice(2) and monkeys(3). Effective irradiated vaccines for the viruses of lymphocytic choriomeningitis(4), St. Louis encephalitis(1), influenza(5) and psittacosis(6) have also been prepared. Relatively few publications have appeared on the preparation of irradiated bacterial vaccines. In 1946 we prepared an irradiated polyvalent bacillary dysentery vaccine which was successful in the active immunization of mice(7) but not of man(8) for reasons other than the method of sterilization. Since the inception of our work we have considered the application of our ultraviolet irradiation technic to the production of a tuberculosis vaccine because of our previous success with the viruses and pathogenic bacteria mentioned above. In the meantime, Olson *et al.*(9) and more recently Sarber *et al.*(10) have prepared ultraviolet killed human type tuberculosis vaccines which have antigenic values at least equal to BCG vaccine on the basis of guinea pig vaccination tests. Paterson *et al.*(11) have prepared an irradiated vole bacillus vaccine which also confers a degree of protection in guinea pigs comparable to that obtained from BCG.

Since all of the reported studies with irradiated tuberculosis vaccines thus far have been confined to the use of guinea pigs, we considered it worthwhile to report the following experiments in which line 1 dba mice were vaccinated with irradiated tuberculosis vaccines. A standard BCG vaccine was used as a positive control vaccine in the comparative active immunity experiments.

Procedure. Mice. Line 1 dba mice weighing 12-15 g obtained from the Roscoe Jackson Memorial Laboratories were used throughout these studies. The mice were fed a diet consisting of Purina dog checkers and housed in glass jars with metal tops.

Irradiated Vaccine. The H37Rv strain of *Mycobacterium tuberculosis* var. *hominis* was used both for the preparation of irradiated vaccines and the challenge inoculation. This strain suspended in 5% gastric mucin consistently produces macroscopic lesions in dba mice when 0.001 mg moist weight is inoculated

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intraperitoneally or subcutaneously(12). The vaccine was prepared by growing the H37Rv strain on Petragnani's media slants incubated at 37°C for 14 days. The cultures were then harvested, weighed, and a suspension in sterile distilled water was prepared and ground for 30 minutes in a ball mill. The suspension was next diluted to contain 1 mg of moist weight of bacilli per cc and filtered through 120 mesh bolting silk cloth in order to remove gross particles and other debris. The suspensions were checked microscopically for purity after staining by the Ziehl-Neelsen technic and irradiated immediately with the Oppenheimer-Levinson type of quartz cell ultraviolet irradiation equipment(1). Careful preliminary titration studies were made to determine the least amount of irradiation necessary to kill the suspensions, and it was found that exposures for 0.5 second would consistently sterilize the vaccines with minimal antigenic destruction. After removing samples for sterility and safety tests, 1:10,000 merthiolate was added as a preservative and the vaccines were stored in the refrigerator at 3-5°C.

Sterility and safety tests. Most lots of irradiated vaccine consisted of 100 to 200 cc quantities of suspension. Each lot of vaccine was tested for sterility by seeding 20 tubes of Petragnani's media, 20 tubes Dubos media and 3 tubes of thioglycollate media with 0.5 cc of undiluted vaccine per tube. Thus 10 to 20% of the total volume was cultured. Simultaneously control samples of suspension before irradiation were tested for viability. All media were incubated at 37°C for 6 months unless growth was apparent sooner. Safety tests were carried out by inoculating 3 guinea pigs weighing 200 to 300 grams with 5 cc of undiluted irradiated vaccine intraperitoneally for each batch of vaccine. The guinea pigs were observed for 3 months and then sacrificed and examined for the presence of lesions. Ten dba mice were also inoculated both subcutaneously (0.5 cc) and intraperitoneally (0.5 cc) with irradiated vaccine mixed with equal parts of gastric mucin. The mice were sacrificed at intervals of 10 to 20 days and examined for lesions.

BCG vaccine. Freshly prepared standard BCG vaccine obtained through the courtesy of Dr. S. R. Rosenthal of the Tice Laboratory of the Chicago Municipal Tuberculosis Sanitarium was used in comparative immunogenic potency tests. The BCG vaccine was less than 5 days old in all experiments.

Results. Comparative active immunization tests were carried out by vaccinating line 1 dba mice with ultraviolet killed H37Rv and freshly prepared, viable BCG vaccines. The irradiated vaccines were at least one month old at the time of the first vaccination, and the BCG vaccine less than 5 days. Groups of mice were given 4 subcutaneous injections of 0.5 cc (0.5 mg) of each vaccine at weekly intervals. The challenge inoculation was given 30 days after the last dose of vaccine and consisted of a saline suspension of a 14-day-old culture of the H37Rv strain grown on Petragnani's media. Different groups of vaccinated and control mice were challenged with 0.025, 0.01 and 0.001 mg moist weight of bacilli suspended in equal parts of 5% gastric mucin inoculated both subcutaneously (0.5 cc) and intraperitoneally (0.5 cc). All animals were sacrificed 30 days after the challenge dose and examined carefully for macroscopic lesions. Impression smears were routinely made of all suspicious lesions and stained by the Ziehl-Neelsen technic.

A summary of the results obtained in several comparative antigenicity tests of ultraviolet killed H37Rv bacilli and living BCG vaccines in vaccinated mice is shown in Table I. Lesions in the Table refer to gross tubercle-like lesions ranging in size from 2 to 4 mm at the site of inoculation, liver, spleen or in the peritoneum. Impression smears of gross lesions in positive mice showed the presence of large numbers of acid-fast bacilli. The results shown in Table I indicate that both the irradiated and BCG vaccines produce a statistically significant† increased resistance to experimental tuberculosis in dba mice. The differences in average number of gross lesions

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† We are indebted to Dr. Herbert Silverstone of Michael Reese Hospital for statistical analyses.

TABLE I. Comparative Antigenicity of Irradiated H37Rv and BCG Vaccines in dba Mice.

Vaccine	Challenge dose, mg	No. mice with lesions / No. mice inoculated	%	Avg No. lesions per mouse
Irrad.	.025	16/36	44.4	1.9
BCG	.025	23/39	59	2.8
Controls	.025	37/37	100	3.1
Irrad.	.01	9/24	37.5	2.4
BCG	.01	13/24	54.2	3.1
Controls	.01	22/22	100	3.3
Irrad.	.001	11/36	30.6	1.6
BCG	.001	20/39	51.3	2.8
Controls	.001	37/37	100	2.8

TABLE II. Antigenicity of Various Lots and Storage of Irradiated Tuberculosis (H37Rv) Vaccines.

Vaccine	Storage time, mo.	Challenge dose, mg	No. mice with lesions / No. mice inoculated	%	Avg No. lesions per mouse
UV Lot #1	1	.025	5/12	41.6	2.2
		.001	4/12	33.3	1.6
#2	1	.025	6/12	50	2.0
		.010	4/12	33.3	3.0
		.001	4/12	33.3	2.0
#3	1	.025	6/12	50	2.5
		.010	5/12	41.6	2.1
		.001	4/11	36.3	2.1
#1	12	.025	6/12	50	2.5
		.010	5/12	41.6	2.0
		.001	4/11	36.3	2.1

in each mouse is not significant, although the maximum number of lesions in the ultraviolet vaccinated mice was 3 compared with 5 in the BCG and control groups. The irradiated vaccine consistently was slightly superior to BCG with the various challenge doses given.

Table II shows the uniformity in antigenic response obtained with 3 different lots of ultraviolet vaccine which were killed by irradiation for 0.5 second. Table II also shows the results obtained in storage experiments of 3 lots of irradiated vaccine after 1 month at 3-5°C as well as one lot (No. 1) which was stored for 12 months without significant loss in potency. All of these vaccines contained 1:10,000 merthiolate as a preservative. Further experiments on storage of the irradiated vaccines are in progress.

Discussion. The results obtained in the present studies indicate that a completely killed ultraviolet H37Rv tuberculosis vaccine gave as good or slightly superior results to BCG in comparative active immunity tests of vaccinated dba mice. These results are in agreement with those obtained by others (9,10) using guinea pigs as the test animal. Our experiments confirm those of Sarber *et al.* (10) that the irradiated tuberculosis vaccine is relatively stable when stored in the refrigerator at 3 to 5°C for several months.

A killed antigenic tuberculosis vaccine offers several advantages over BCG. First, since BCG contains living organisms to which a preservative cannot be added there is always the potential hazard of contamination with a pathogen. Furthermore, Vorwald *et al*(13) have recently reported that BCG produced a progressive and fatal tuberculosilicosis in silicotic guinea pigs. Finally, the known instability of BCG is a great disadvantage for general use of the vaccine.

No attempt was made to test the effectiveness of the irradiated vaccine against heterologous strains of virulent tubercle bacilli.

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No attempts were made to test the antigenicity of ultraviolet-killed BCG or heat killed virulent tubercle bacilli vaccines because of the ineffectiveness of such vaccines previously reported by others(9,10).

Summary. Several lots of ultraviolet-killed virulent human type tubercle bacilli (H37Rv) vaccine were prepared which were equal or slightly superior in antigenic potency to a standard BCG vaccine on the basis of dba mouse vaccination tests. The irradiated vaccine preserved with 1:10,000 merthiolate was stable and retained its immunogenicity for as long as 12 months after preparation.

Received November 2, 1950. P.S.E.B.M., 1950, v75.

Amount of Luteal Tissue Required for the Maintenance of Pregnancy in the Rat.* (18324)

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Gustav Born first suggested that the corpora lutea maintain the uterus in a condition compatible for pregnancy(1). Fraenkel and Cohn(2), Fraenkel(3,4) and Corner(5) confirmed this hypothesis in the rabbit. Harris (6) and Parkes(7) found that ovariectomy always produced resorption or abortion in mice. Loeb(8) showed that pregnancy in guinea pigs was terminated if the corpora were removed before the end of the second day but was uninterrupted if removed after the end of the second day. That ovariectomy causes abortion in a high percentage of guinea pigs was demonstrated by Herrick(9), but ovarian transplants reduced the possibility of abortion. Courrier(10) suggested that endocrine secretions of the placenta may prevent some abortions which would normally be expected. Hartmann(11) suggested that man, the guinea pig, the horse, and the monkey are exceptions to the dependence of successful gestation on the presence of luteal secretions. Similar work on the rat by Harris and Pfiffner(12), Nelson and Haterius(13), Johnson and Challans(14,15), Pencharz and Long(16), Hain(17) and Zeiner(18) demonstrated that in a majority of the cases pregnancy was terminated within 48 hours after bilateral ovariectomy. Preliminary studies of lutein tissue required to maintain pregnancy in the rat done in our laboratories, indicate that the rat is able to parturate having only

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* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and in part by a grant from the Division of Research, Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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TABLE I. Effects of Corpora Lutea Ablation on Maintenance of Pregnancy.

Group	No. of animals	Corpora wt, avg range, mg	Adrenal wt, avg range, mg	No. of fetuses avg range	No. of placental sites avg range
Normal	8*†	4.4(4.0-5.3)	63.1(56.8-77.0)	10(5-12)	10+(5-12)
2 c. 1, 8th day	10*‡	5.1(3.5-6.8)	61.3(52.0-78.5)	9(3-15)	10 (5-15)
2 c. 1, 8th day	3‡	2.4(2.3-2.5)	52.3(49.0-58.5)	—	9 (7-12)
1 c. 1, 8th day	9‡	2.5(1.0-3.0)	56.8(51.0-69.0)	—	9 (4-11)
1.5 c. 1, 8th day	6‡	3.3(2.0-4.3)	58.6(49.5-67.0)	—	11 (9-13)
1 c. 1, 15th day					
Group A	6‡	4.7(4.5-5.0)	73.3(51.5-112.0)	—	11 (7-14)
Group B	4*‡	5.1(4.5-6.0)	56.9(52.5-61.0)	3(1-5)	8 (5-10)
Group C	5*‡	5.3(5.0-6.0)	65.8(50.0-92.0)	4(1-7)	10 (8-13)
2 c. 1, 8th day A/P 12	5*†	4.7(4.0-5.0)	37.6(33.0-44.0)	8(5-9)	9 (8-10)

* Animals in these groups parturated or had living young in uteri at autopsy.

† Autopsied on 20th day of pregnancy.

‡ Autopsied post partum.

two corpora lutea (unpublished data).

The purpose of this paper is to present more extensive data and to show the importance of the corpora lutea of pregnancy in the rat.

Methods and materials. The rats used were 90- to 120-day-old females of the Holtzman strain. Day one of pregnancy was considered the day following insemination. The experiment was divided into 6 sections: (1) normal pregnant rats; (2) rats from which all corpora except 2 were removed on the eighth day of pregnancy; (3) rats from which all corpora except one were removed on the eighth day of pregnancy; (4) rats from which all corpora except one and part of another were removed on the eighth day of pregnancy; (5) rats from which all corpora except one were removed on the 15th day of pregnancy; and (6) rats from which all corpora except 2 were removed on the eighth day of pregnancy and the pituitary on the 12th day. The eighth day of pregnancy was chosen as the day for the removal of the corpora in the first sections of the study because by that time implantation of the fetuses is underway.

The hypophysectomized group having 2 corpora was autopsied on the 20th day of pregnancy and all other groups as soon as possible after parturition or at the time of resorption as indicated by vaginal bleeding. The majority of the data was collected on the 21st and 22nd day of pregnancy. At this time very little involution of the corpora of pregnancy has taken place as shown by Biddulph, Meyer and McShan(19). The pituitaries were removed by means of the parapharyngeal approach and immediate postoperative care was limited to the feeding of dextrose and bread. Ablation of the corpora lutea was performed by baring the ovaries one at a time and removing the corpora with sharp pointed forceps after first rupturing the bursa. Bleeding was kept at a minimum by occluding the ovarian circulation with a hemostat fixed on the mesovarium and swabbing the surface of the ovary from time to time with Thromboplastin.[†]

Results and discussion. Data collected from 8 intact rats on the 20th day of pregnancy provide normal weight values for corpora lutea and adrenal glands, and for the number of fetuses and placental sites (Table I).

The first experimental data were from rats in which all but 2 corpora lutea were removed on the eighth day of pregnancy. Ten of the 13 rats used in this group parturated and in the remaining 3 the fetuses were resorbed by the 12th day. The reabsorption may have been due to an interference with the blood supply to the corpora lutea at the time of operation as the corpora did not increase in size.

Nine animals were in the group in which all but one corpus were removed on the eighth

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[†] E. R. Squibb and Sons, New York.

day of pregnancy (Table I). Pregnancy failed in all of these. Resorption was indicated by vaginal bleeding which occurred 24 to 36 hours after treatment and was observed to last only a few hours.

In the work just described, one rat parturated which had only one corpus and part of another present at autopsy. Since 2 corpora will maintain pregnancy, although one will not, it was considered possible that the progesterone level required to maintain pregnancy might be produced by one corpus and part of another. A group of 6 rats had all corpora but one and part of another removed on the eighth day of pregnancy, but in none did pregnancy continue to beyond the 13th or 14th day of gestation, at which time the majority of these rats were autopsied. Considering the data presented thus far, it is clear that to ensure a successful pregnancy in the rat at least 2 corpora must be present.

Fraenkel(2-4) working with rabbits, demonstrated that the removal of all corpora lutea on or after the 20th day of pregnancy did not terminate gestation. Assuming that the 15th day would be an equivalent time in the gestation period of the rat, it was thought advisable to secure data from rats in which all corpora but one were removed on the 15th day. Fifteen rats were so treated and on the basis of the results the data have been placed in 3 categories. The first one consists of those in which pregnancy was interrupted soon after the operation; the second, those which gave birth to some of the fetuses but had others present in the uterine horns in a partially resorbed condition; and the third category includes those rats which gave birth to all of their young. The size of the litters for the last 2 categories as given in Table I are small because of cannibalism on the part of some of the rats.

The first group included 6 rats in which the average weight of the adrenals was 73.3 mg per pair as compared to the normal value of 63.1 mg. This increase in weight may reflect the metabolic stress caused by the resorption of the fetuses. The data in the second group were gathered from 4 rats while data from 5 rats made up the third group.

It is interesting to note that although the first group had a greater average number of placental sites than the other 2 groups, the placentae, as possible supplementary sources of progesterone, were not capable of maintaining pregnancy (Table I). Selye, Collip and Thomson(20) have shown that in pregnant rats which are castrated and the embryos removed the placentae can produce enough progesterone to maintain the endometrium in a state which will support the placentae. They, however, cannot secrete enough progesterone to maintain the endometrium in a condition which will support both the placentae and the embryos. Therefore, the secretory capacity of the single corpus may be the determining factor whether or not pregnancy will continue uninterrupted.

It has been shown by Pencharz and Long (21), Selye, Collip and Thomson(22) and Meyer and Shipley(23) that rats which are hypophysectomized on the 12th day of pregnancy will go to the end of the gestation period without difficulty, but they may not parturate. Pregnancy is terminated in those hypophysectomized before the 10th day of gestation. This points to the placenta as a source of luteotrophin for the maintenance of the corpora lutea. That the placenta in the rat is a source of luteotrophin was demonstrated by Astwood and Greep(24) who maintained the corpora of pseudopregnancy in hypophysectomized rats by injecting saline suspensions of fresh rat placental tissue twice daily.

It was not known whether this placental-corpora lutea relationship would be effective in rats where all except 2 corpora were removed on the eighth day and hypophysectomized on the 12th day of pregnancy. To test this hypothesis, data from 5 animals treated

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20. Selye, H., Collip, J. B., and Thomson, D. L., *Endocrinology*, 1935, v19, 151.

21. Pencharz, R. E., and Long, S. A., *Am. J. Anat.*, 1933, v53, 117.

22. Selye, H., Collip, J. B., and Thomson, D. L., PROC. SOC. EXP. BIOL. AND MED., 1933, v30, 589.

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in this manner were collected; in all pregnancy continued to the time of autopsy.

The average weights of the corpora of the hypophysectomized animals are much like those of the normal animals which indicate that the corpora are not grossly affected by the removal of the pituitary. Also, the average weights of the corpora in this group were of the same order as the average weights of those in the group having only 2 corpora from the eighth day of pregnancy. The corpora in this group were maintained by the luteotrophin produced by the placentae. However, the adrenals were greatly reduced in size which demonstrates the effect of the complete lack of a trophic hormone on its end organ (Table I).

The experimental design included procedures for studying the correlation between adrenal weight and the maintenance of pregnancy. There is evidence from several sources, among them Beall(25,26), Beall and Reichstein(27), Riddle and Lahr(28), VanHeuverswyn, Collins, Williams and Gardner(29) and Zarrow, Hisaw and Bryans(30), that there are adrenocortical compounds having a progesterone-mimetic effect. On the basis of this evidence it was thought possible that in pregnant rats where the corpora lutea are reduced in number the adrenals might respond by hypertrophy and hypersecretion and thus maintain pregnancy. This hypothesis is not supported by the data as the adrenals do not show any generalized increase in weight over the normal (Table I). However, this does not discount the possibility of a hypersecretion without an accompanying hypertrophy which could not be detected by the methods employed in this study.

Conclusions. The albino rat of the Holtzman strain was used to determine the minimum number of corpora lutea required to maintain pregnancy. It was found 2 corpora will maintain pregnancy from the eighth day of gestation, but one corpus will not, nor will one corpus and part of another. In animals where all corpora but one were removed on the 15th day of pregnancy, two-thirds either had litters of normal fetuses or some of the fetuses were normal while others were being resorbed. All of the fetuses of the other one-third of the animals were being resorbed at the time of autopsy. Pregnancy was maintained in a group of animals in which all but 2 corpora were removed on the eighth day and hypophysectomized on the 12th day of pregnancy.

There is no evidence that the placentae are adequate supplementary sources of progesterone in those groups where the corpora were reduced below two at the eighth day, since pregnancy was terminated in all cases. However, when the corpora were reduced to one on the 15th day of pregnancy, pregnancy was terminated in only one-third of the group which may indicate that the placenta is a more effective supplementary source of progesterone in the latter third of pregnancy. Reduction in the number of corpora to two per rat does not cause a hypertrophy of the adrenal glands.

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Received November 1, 1950. P.S.E.B.M., 1950, v75.

Effects of Cortisone and ACTH Therapy on Eosinophils of the Bone Marrow and Blood. (18325)

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(Introduced by W. Antopol.)

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Recent reports have shown that cortisone and ACTH decrease markedly the number of eosinophils in the peripheral blood(1-3). The importance of this finding is reflected in the extensive use of this response as a guide to the therapeutic effectiveness of both cortisone and ACTH(2). Aside from its relation to adrenal cortex function(4), the mechanism of the eosinopenia is at present unknown. The effects of cortisone and ACTH on the eosinophils of both the bone marrow and peripheral blood are reported herein.

Methods and procedure. Eosinophil counts were performed on bone marrow and peripheral blood obtained from patients with non-hematological diseases. The counts were done according to the propylene glycol-phloxine method described by Randolph(5). The studies were carried out before and during therapy, administered according to the therapeutic requirement.* Six patients received cortisone and 1 received ACTH. The amount of bone marrow aspirated from the sternum was slightly in excess of 0.1 cc in order to supply sufficient material for both eosinophil count and slide preparations. The peripheral blood counts were performed on blood from the finger tip, obtained a few minutes prior to sternal puncture.

Results. The eosinophil counts of the peripheral blood and bone marrow before and during therapy are shown in Table I. All 7 subjects showed significant decreases varying from 64 to 100% in the blood esosinophil counts after cortisone or ACTH therapy whereas the bone marrow eosinophilic cell counts showed consistent increases ranging from 42 to 372%. Differential cell counts of the bone marrow showed no significant alterations after therapy.

Discussion. It is apparent from these results that at the time of marked reduction in the peripheral blood eosinophils during cortisone or ACTH therapy, there is no decrease in

TABLE I. Eosinophil Counts of Blood and Bone Marrow of 7 Patients Before and During Either Cortisone or ACTH Therapy.

Subject	Sex	Age	Diagnosis	Drug	Day of therapy studied	Total dose, mg	Blood eosinophil count (per cu mm)			Bone marrow eos. count (per cu mm)		
							Before therapy	After therapy	% change	Before therapy	After therapy	% change
A.G.	F	68	Rheum. arthritis	Cortisone	12	1100	81	30	— 64	3440	16250	+372
L.G.	F	35	” ”	”	2	200	198	69	— 65	1469	2600	+ 76
A.Y.	F	57	” ”	”	7	1000	22	0	—100	406	575	+ 42
P.K.	F	26	” ”	”	7	1000	106	25	— 77	656	1965	+198
A.H.	M	59	” ”	”	7	1000	500	120	— 76	1750	3049	+ 74
H.D.	M	45	Regional ileitis	”	6	1600	75	0	—100	731	1275	+ 74
B.G.	M	54	Rheum. arthritis	ACTH	4	240	131	6	— 95	837	1435	+ 71

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* These patients were treated by Dr. Jack Dordick.

the number of eosinophilic cells in the bone marrow. This is in accord with the observation that cortisone administration does not produce any diminution in eosinophils in the femoral marrow of mice(6).

The apparent increase in the eosinophilic cells of the bone marrow indicates that cortisone and ACTH may inhibit delivery of eosinophils from the bone marrow to the peripheral blood. However, the differential counts indicate no significant alteration in the percentage of the eosinophilic cells in relation to the nucleated cells of the bone marrow. It appears most probable that cortisone and ACTH increase the rate of removal of eosinophils from the blood without affecting their production by the bone marrow.

Summary. Eosinophil counts were performed on blood and bone marrow of 7 patients before and during either cortisone or ACTH therapy. At a time of marked diminution in the eosinophils of the peripheral blood, the eosinophilic cells of the bone marrow were not decreased.

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Received November 8, 1950. P.S.E.B.M., 1950, v75.

Influence of Tumors on Liver Concentration of Radioactive Stilbamidine.* (18326)

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(Introduced by J. H. Lawrence.)

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During the course of studies with radioactive stilbamidine, certain findings have appeared that are of sufficient interest to warrant a preliminary report. Kopac(1) reported that stilbamidine destroyed the neoplastic cells in cultures of rat mammary adenocarcinoma and transplanted lymphosarcoma. Snapper(2) further studied this drug and found inclusion bodies containing stilbamidine in the cytoplasm of plasma cells in patients with multiple myeloma. Accordingly, C^{14}-labeled stilbamidine was prepared and its metabolic distribution in normal mice and a patient with multiple myeloma was undertaken(3,4). These experiments established that the oxidation to $C^{14}O_2$ of stilbamidine by the body is negligible.

These studies suggested the possibility of investigating the localization and distribution of activity in mice with various types of tumors. Accordingly, normal and tumor bearing mice were injected intraperitoneally with 0.4 mg (4.50 x 10^6 dis/min) of stilbamidine-C_2^{14} dissolved in 0.05 ml saline. The C^{14}-labeled stilbamidine used for this experiment was synthesized by Dr. J. C. Reid(5).

Procedure. Ninety-six hours after injection, the mice were killed with ether and autopsied. Liver, spleen, kidneys, pancreas and tumors were removed, dried in vacuum, weighed and burned over copper oxide and the resulting carbon dioxide was converted to barium carbonate and the radioactivity measured with a proportional counter(6).‡ The mice were kept in separate cages designed to prevent contamination of food and water with excreta.

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* Supported in part by the Atomic Energy Commission.

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TABLE I. Activity Found in the Livers of 16 Tumor Mice 96 Hours After Injection of Labeled Stilbamidine.

Strain	Sex	Age (mo.)	Mouse wt, g	Tumor wt, wet, g	Liver wt, dry, g	Total liver activity (% of inj. dose)	Type of tumor
A	♂	6	24	4.45	.272	40	Sarcoma
A	♂	6	23	2.09	.265	23	Mammary
A	♂	5	23	4.45	.321	23	Sarcoma
A	♂	5	21	3.39	.310	30	,,
A	♂	5	22	4.26	.299	31	,,
A	♂	5	24	4.76	.326	31	,,
A	♂	6	21	large	.176	19	,,
A	♂	6	21	large	.184	21	,,
dba	♂	10	24	2.27	.407	19	Melanoma
,,	♂	8	32	4.67	.559	12	,,
,,	♂	8	29	7.06	.547	11	,,
McDowell	♀	6	26	0.06	.430	12	Lymphatic leukemia
,,	♀	18	20	0.30	.296	35	Lymphatic leukemia
,,	♀	6	19	0.16	.258	33	Lymphatic leukemia
C57	♂	6	30	0.58	.573	24	Myelogenous
,,	♀	6	36	6.03	.659	41	Mammary

TABLE II. Activity Found in the Livers of 20 Normal Mice 96 Hours After Injection of Labeled Stilbamidine.

Strain	Sex	Age (mo.)	Mouse wt, g	Liver wt, dry, g	Total liver activity (% of inj. dose)
A	♂	10	24	.356	2.5
A	♂	12	27	.496	7.7
A	♂	6	24	.535	7.7
A	♂	6	24	.539	7.5
A	♂	5	20	.336	4.4
A	♂	5	22	.380	4.8
A	♂	5	22	.389	2.0
A	♂	5	22	.408	3.0
Bagg	♂	10	32	.512	5.1
,,	♂	10	34	.513	7.5
,,	♂	10	32	.579	6.4
dba	♀	18	28	.336	8.0
,,	♂	8	23	.473	4.6
,,	♂	8	25	.517	5.0
C57	♀	6	23	.406	30.
,,	♀	6	21	.420	17.
,,	♂	6	24	.459	24.
,,	♂	6	24	.443	34.
,,	♂	13	32	.575	36.
,,	♂	13	32	.57	30.

Results. Tables I and II show the total concentration of radioactivity present in the livers of normal and tumor mice 96 hours after injection. The total liver activity is given as percent of injected dose. It is evident that very much higher and distinctly abnormal concentrations of radioactivity are present in the livers of the tumor-bearing mice as compared to the controls for the *A* and *dba* strains. With the number of animals used no correlation between tumor weight and liver concentration of radioactivity can be established. However, there appears to be some correlation between mouse weight and liver concentration of radioactivity. Thus, there is a small increase in liver concentration in the normal group with increasing mouse weights. Conversely, there appears to be a drop in liver concentration in tumor mice with increasing mouse weights. The number of such mice is inadequate to definitely establish this correlation. The 3 mice with lymphatic leukemia were found to have elevated levels of radioactivity in the liver, but the significance of this group is not known since no control determinations have been made for mice of this strain.

It is of considerable interest that the normal C-57 strain mice have liver concentrations of radioactivity in the same range as mice with neoplasms. Whether this is unique for mice which develop spontaneous nonepithelial neoplasms or whether it is coincidental remains for further study. Here increasing mouse weights appear to be associated with increasing liver concentrations of radioactivity following the pattern of the other normals in this respect. More mice with tumors will need to be studied before the effect of tumors on liver concentration in this strain can be known.

The specific activity of liver for the tumor mice averaged 3,464 dis./mg/min. and for the strains A, dba and Bagg controls 498 dis./mg/min. For the normal strain C57 mice the specific activity averaged 2,726 dis./mg/min. No definite correlation could be established between tumor weight or mouse weight and specific activity.

The concentrations of activity in the kidneys and spleens of the tumor mice averaged 7.5 and 0.24% of injected dose respectively and are a little greater than in the normals, but the differences are not significant for the number of animals studied to date. The concentration of radioactivity in the pancreas was found to be intermediate between spleen and kidney and is in the same range for both control and tumor mice. None of the tumors contained more than traces of activity which compares with Snapper's qualitative results in which he found no evidence of localization of stilbamidine in transplantable lymphosarcoma and mammary carcinoma(7).

Kopac has shown that stilbamidine dissociates protamine-nucleate complexes with the release of partially denatured protamine molecules and the formation of an insoluble stilbamidine-nucleate complex(8). Snapper and his group have presented evidence that stilbamidine is capable of specifically combining with ribose nucleic acid in the cytoplasm of myeloma cells(2). Kelly and Jones(9) have found a considerable increase in turnover rates of desoxypentose nucleic acid in the liver, spleen and kidneys of tumor mice with transplanted mammary cancer as compared with turnover rates in normal mice. The behavior of ribose nucleic acid in this regard is not known.

It seems possible, then, that stilbamidine precipitates in the liver cells as an insoluble stilbamidine-nucleate complex and the increased amounts present in tumor-bearing animals are related to increased formation of certain nucleic acids in these livers. Studies are being made to determine the chemical natur of the stilbamidine complex or derivative that has concentrated in these livers and in what elements of the liver this material is located.

In a patient with multiple myeloma, who was injected with C^{14}-labeled stilbamidine, it was found that an unexpectedly high concentration of radioactivity was present in the liver at autopsy three months later(4). While this fits the pattern of the mouse results, more studies of humans with and without malignant disease will be necessary before significance can be attached to this isolated finding.

Summary. A series of normal and tumor mice were injected with stilbamidine-amidine-C_2^{14}. After 96 hours organs were removed and analyzed for radioactivity. Much higher and distinctly abnormal concentrations of C^{14} were found in the livers of tumor-bearing A and dba strain mice as compared to the controls. Some data for C57, Bagg and McDonnell strains are also given. The relation of this phenomenon to possible abnormalities in nucleic acids in tumor-bearing mice is discussed.

The authors with to thank Professors J. H. Lawrence, H. B. Jones and M. Calvin for their interest and help in this work and Mr. Saburo Ikeda for radioactive measurements.

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Received November 3, 1950. P.S.E.B.M., 1950, v75.

Effect of Dosage on Length of Incubation Period of Aster-Yellows Virus in its Vector. (18327)

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The vector of aster-yellows, *Macrosteles divisus* Uhler, may become infective through feeding on a diseased plant(1) or by receiving an injection of juices from leafhoppers that had fed on a diseased plant some days previously(2). In either case, the vector is unable to transmit virus to healthy plants immediately after acquiring it. An interval of several days, often referred to as an incubation period of the virus in the leafhopper, must elapse between the time when the insect first acquires virus and the time when it can first transmit virus to healthy plants. An attempt was made to determine whether or not there was a relationship between the amount of virus injected and the length of the incubation period in the leafhopper.

Procedure. One hundred infective leafhoppers were crushed at 0°C in a glass micromortar and the pulp diluted to 10^{-1} and 10^{-3} with 0.25 M neutral buffered saline. Two hundred non-viruliferous insects, immobilized by a temperature of 0°C, were injected with each dilution. The volume of inoculum, 1/8000 cc per insect, was delivered by a specially calibrated microsyringe. The surviving injected vectors were kept under controlled light (1500 foot candles), constant wind velocity and humidity, at $25°C \pm 0.5°C$. Non-injected insects from the same stock served as controls. The leafhoppers were caged on young asters in colonies of 5 and transferred daily to new sets of healthy plants. With a dilution of 10^{-1} the shortest incubation periods of 3 infective colonies were 11, 14, and 15 days, respectively. With a dilution of 10^{-3} the minimal incubation periods were 24, 28, and 38 days, respectively. A similar relationship was described earlier for the wound-tumor virus(3) and for the animal viruses causing rabbit papilloma(4) and encephalomyelitis of mice(5). In the latter instance the incubation period was used as a measure of virus activity.

The results of the experiments with aster-yellows virus can be interpreted on the same basis as those with animal viruses, that is, on the fact that multiplication of a small amount of virus would take a longer time to render the insects infective than multiplication of a greater amount. This interpretation is in full agreement with earlier evidence on the multiplication of this plant virus in its insect vector(6-9). It is expected that more extensive experiments will show that the correlation, between amount of virus injected and length of incubation period of virus in the leafhopper, will provide a fairly accurate measure of aster-yellows virus concentration in leafhopper juice samples.

Summary. Juices from viruliferous *Macrosteles divisus* Uhler were diluted to 10^{-1} and 10^{-3} with saline and 1/8000 cc per insect was injected into non-viruliferous leafhoppers. The infectivity tests of injected vectors under controlled environmental conditions showed that with a dilution of 10^{-1} the shortest incubation periods were from 11 to 15 days. With a dilution of 10^{-3} the minimal incubation periods were from 24 to 38 days. The results can be interpreted on the basis of the fact that multiplication of a small amount of virus would take a longer time to render the insects infective than multiplication of a greater amount of the aster-yellows virus.

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Received November 3, 1950. P.S.E.B.M., 1950, v75.

Inhibition of Synthesis of Vitamin B_{12} and of Riboflavin by 1,2-Dichloro-4,5-diaminobenzene in Bacterial Cultures. (18328)

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It has been shown recently(1) that 1,2-dichloro-4,5-diaminobenzene is a rather selective poison, in that it inhibits the growth of many kinds of living things which can synthesize riboflavin or vit. B_{12}, or both, and is not harmful to those species which cannot form either of these substances. The demonstration arose from the realization that both of these vitamins probably had a common precursor, namely 1,2-dimethyl-4,5-diaminobenzene, and that a properly constituted analogue of this metabolite, such as 1,2-dichloro-4,5-diaminobenzene should exert such a selective action because it would inhibit the synthesis of these two vitamins in those cells which were endowed with the metabolic machinery for conducting such syntheses. By choosing a common metabolic precursor of two vitamins concerned intimately in cell division, rather than a precursor of one such substance, it was postulated that an agent might be realized which would act even in the presence of the products of the inhibited reactions. This actually proved to be the case with 1,2-dichloro-4,5-diaminobenzene because both riboflavin and vit. B_{12} did not prevent its action.

The working hypothesis just described involves some assumptions because 1,2-dimethyl-4,5-diaminobenzene, the postulated common precursor of both vitamins, has not been demonstrated to be the actual starting point for such biosyntheses. The only evidence in favor of such a view has been that this substance can be recognized in the formulations of these two vitamins, and that it has a trace of riboflavin activity for *Lactobacillus casei,* and of vit. B_{12} potency for 2 other microorganisms(1). The fact that it will antagonize the growth-inhibiting effects of 1,2-dichloro-4,5-diaminobenzene in a competitive fashion has suggested that the latter substance is exerting its toxicity by interference with the functioning of the dimethyldiamine for the synthesis of riboflavin and vit. B_{12}. The present investigation was undertaken in order to determine whether any evidence of a more direct nature for this postulate could be found.

The 1,2-dichloro-4,5-diaminobenzene inhibited the formation of vit. B_{12} and of riboflavin by growing cultures of *Bacillus megatherium* strain WRRL B-938.† When this organism was cultured aerobically in flasks at 30° for 48 hours, in the riboflavin-free Landy-Dicken medium(2) previously described for testing toxicity of this compound (1) half-maximal inhibition of growth was produced by 7-8 μg of the dihydrochloride per cc. For measurement of any possible effect on the formation of riboflavin and of vit. B_{12} in such cultures, an amount of the analog was chosen which was just insufficient to cause detectable inhibition of growth. This, and graded dilutions lower than this, were added to the medium before inoculation. After seeding and growth had occurred, microbiological assays for the two vitamins in question were performed on the heated, and suitably diluted cultures. Riboflavin was determined with the aid of *Lactobacillus casei,* and vit. B_{12} with *Euglena gracilis*(3). Results of a typical experiment are shown in Table I. There it can be seen that quantities of the analog which were too small to cause inhibition of growth, reduced the concentration of these 2 vitamins which this organism formed. The reduction in vit. B_{12} was greater than in riboflavin. A situation similar to this inter-

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* With the technical assistance of N. Smith and E. A. Singer.

1. Woolley, D. W., *J. Exp., Med.,* 1951, v93, 13.

† This organism was very kindly supplied by Dr. D. Perlman of E. R. Squibb and Sons, Inc.

2. Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.,* 1942, v27, 1086.

3. Hutner, S. H., Provasoli, L., Schatz, A., and Haskins, C. P., *Proc. Am. Phil. Soc.,* 1950, v94, 152.

TABLE I. Effect of 1,2-Dichloro-4,5-diaminobenzene Dihydrochloride on the Synthesis of Vitamin B_{12} and of Riboflavin by *B. megatherium.*

Analog in the growth medium, μg per cc	Light transmission (growth) %	Vit. B_{12} synthesized, mμg per cc	Riboflavin synthesized, μg per cc
0	50	.083	.14
10	87	—	—
5	53	.045	—
3	52	—	.10
2	43	.038	—
1	42	.060	—

ference with vit. B_{12}- and riboflavin-production was found by Miller(4) who showed that sulfanilamide and its congeners reduced the amount of folic acid which cultures of *Esherichia coli* formed. In this case, however, the degree of inhibition was greater.

When 1,2-dimethyl-4,5-diaminobenzene was added to growing cultures of *B. megatherium,* (in the absence of the dichloro-analog), a stimulation in synthesis of vit. B_{12} was observed. Thus, when 10 μg of this postulated metabolic precursor were present per cc during growth under the conditions just defined, approximately twice as much of the vitamin was formed as in the unsupplemented basal medium (0.14 millimicrogram compared to 0.083 millimicrogram). Although the dimethyldiamine has a slight activity as vit. B_{12} for *E. gracilis*(1), control experiments showed that the amount employed in the test just described was insufficient to detect. The increase observed was therefore probably due to newly synthesized vit. B_{12}.

When these experiments were completed, the publication of Hendlin and Ruger(5) appeared which showed that additions of cobalt ions to bacterial culture media increased the amount of vit. B_{12} synthesized by various microorganisms grown in them. Because cobalt salts had not been added to the basal medium used in the present investigation the experiments were repeated with cobalt nitrate (10 μg per cc) in the culture fluid. Under these conditions the organism formed 0.45 millimicrogram of vit. B_{12} per cc. The presence of 5 μg of the dichlorodiaminobenzene dihydrochdoride reduced this production to 0.07 millimicrogram of vit. B_{12}, and 2 μg of this same compound resulted in the appearance of 0.23 millimicrogram of the vitamin. In the presence of cobalt therefore, the inhibiting effect of this analog on synthesis of this vitamin was magnified. Cobalt ions did not detectably alter the growth-retarding potency of the dichlorodiamine.

Summary. The amounts of vit. B_{12} and of riboflavin which were formed during growth of *Bacillus megatherium* were decreased by 1,2-dichloro-4,5-diaminobenzene when the latter was present in quantities insufficient to inhibit growth. 1,2-Dimethyl-4,5-diaminobenzene caused stimulation of the production of vit. B_{12}. These facts have been viewed as being compatible with the previously expressed postulates that the dimethyldiamine is a precursor of riboflavin and of vit. B_{12}, and that the dichloro-analog exerts its selective toxicity, at least in part, by inhibition of biosynthetical processes in the formation of these vitamins.

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4. Miller, A. K., PROC. SOC. EXP. BIOL. MED., 1944, v57, 151.

5. Hendlin, D., and Ruger, M. L., *Science,* 1950, v111, 541.

Received November 8, 1950. P.S.E.B.M., 1950, v75.

A Constant Registry Autoradiographic Technic. (18329)

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(Introduced by William C. Young.)

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During recent years several autoradiographic methods, particularly adapted for certain tissues and certain radioactive substances, have been developed. However, previous to the work of Hoecker and Roofe (1), none had been developed for bone which permitted exact correlation between the microscopic structure of bone and the alpha-particle track image of the radioactive substance in the bone after the bone section was removed during processing. In order to study the distribution and concentration of radium metabolically deposited in bone, it is necessary that certain very definite requirements be fulfilled by the autoradiographic technic. (1) It must permit the bone sections to be removed from the nuclear track emulsion during photographic processing and thus prevent the bone section from coming into contact with the processing solutions. (2) It must permit the bone sections to be removed from the nuclear track emulsion during the making of photomicrographs of the alpha-particle track pattern. (3) It must permit the bone sections to be returned exactly to their original positions on the nuclear track plate at any desired time.

The undecalcified bone sections are cut on a special apparatus (Fig. 1 of ref. 2) and then mounted on one end of 1 inch x 3 inch plastic cover slips* with Canada balsam. Before the bone sections are mounted, a rectangular section ¼ inch x 1 inch is cut out of the center of each cover slip in such a manner that the long axis of the cutout is parallel to the long axis of the cover slip. The purpose of this cutout is to concentrate the bending of the cover slip in the region of the cutout portion and thus relieve the strain on the bone sections when the cover slip is bent. Several small holes are punched in the end opposite the bone sections to facilitate attachment of the cover slip to the nuclear track plate by a special wax consisting of equal parts bees wax and rosin. Eastman nuclear track plates, type NTA, with an emulsion thickness of 50 μ are used. The plastic cover slip is securely clamped to the plate at its mid-point with the bone sections in contact with the emulsion. The wax is first melted directly onto the emulsion side of one end of the nuclear track plate with a pencil-type soldering iron; that end of the cover slip containing the small holes is then pressed down into the melted wax, and more of the wax is melted onto the top of the holes. When the bone sections are mounted in this manner on the nuclear track plate, they may be removed from the emulsion surface by lifting the end of the cover slip to which the bone sections are attached away from the nuclear track plate and thus bending the cover slip in the region of the rectangular cutout. When the cover slip is released, the bone sections will return exactly to their original positions if the temperature at the time they are returned is the same as at the time of the original contact. During exposure it is necessary for the bone sections to be in close contact with the emulsion. This contact is best achieved by placing a one-inch cube of lead on top of that end of the cover slip containing the bone sections. It is desirable to obtain a more equal distribution of weight by placing a glass slide between the cover slip and the lead weight. The exposing autoradiographs are then placed in a light-tight box until time for development.

These plates are processed in a specially designed processing tank consisting of 3 separate compartments, each measuring 6⅛ inch x 2¾ inch x 7¾ inch. A new hard rubber

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1. Hoecker, F. E., Roofe, P. G., *Radiology*, 1949, v52, 856.

2. Roofe, P. G., Hoecker, F. E., Vorhees, C. D., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 619.

* The plastic cover slips obtained from Charles F. Hubbs, New York City.

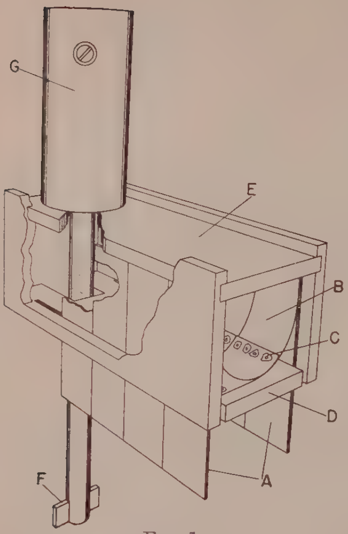

Fig. 1.

Autoradiograph processing unit consisting of nuclear track plate carrier and agitator; nuclear track plates and cover slips in position for processing. A. Immersed portions of the nuclear track plates. B. Bent plastic cover slip. C. Bone sections mounted on end of cover slip. D. Plate carrier. E. Base of motor-driven agitator fitted into grooves extending longitudinally in side pieces. F. Paddle of motor-driven agitator. G. Agitator motor.

storage battery case has been used satisfactorily. On the inner sides of each compartment are vertical projections extending from the bottom to a point 2½ inches below the top. Each compartment is filled with a processing solution up to a level just below the termination of the side projections. The plate carrier is supported just above the level of the processing solutions by the vertical side projections. The plate carrier (Fig. 1) consists of a flat rectangular piece of plastic of such dimensions as to allow it to fit down into the tank compartments and be supported by the side projections. Two slots, each ¼ inch from one of the longitudinal edges and parallel to it, extend from one end of the plate carrier to within 1½ inches of the other. The nuclear track plates fit into these slots during the processing. In the center of that end of the plate carrier into which the slots do not extend is cut a notch through which the shaft of a motor-driven agitator passes. In order to place an exposed nuclear track plate in one of the slots of the plate carrier, the attached plastic cover slip is bent away from the nuclear track plate to a position perpendicular to the plate. The plate is then placed in one of the slots in such a manner that its long axis is perpendicular to the carrier surface while the bent cover slip is held against the upper surface of the carrier, and one half of the track plate extends below. (Fig. 1). Thus the bone sections are protected from contact with the processing solutions by the plate carrier, and that half of the plate extending below the carrier is immersed in the solutions. A plastic side piece two inches high extends perpendicularly along each longitudinal edge of the plate carrier. A groove for the base of a motor-driven agitator extends the length of each side piece ½ inch below its top edge. The function of the side pieces is to help maintain the nuclear track plate in a vertical position during the processing and to support the base of the motor-driven agitator.

The agitator consists of a motor-driven plastic rod with a paddle attached to its free end. (Fig. 1). The small motor is mounted on a flat piece of plastic which fits into the grooves in the side pieces described above. When in position in the grooves the plastic base serves to prevent the nuclear track plates from slipping up because of the tension produced by the bent cover slips. When the plate carrier and agitator are in proper position in the tank, the rod and paddle of the agitator extend down three inches below the nuclear track plates.

The nuclear track plates are processed in accordance with the recommendations of Eastman Kodak Company. However, before the developed and cleared nuclear track plates are washed, they must be removed from the plate carrier. It is necessary that the bone sections neither come in contact with the wet, soft emulsion or be exposed to the running water. To prevent this, an open side tubing clamp is clamped over the nuclear track plate and cover slip immediately in front of the wax. The cover slip, which has thus far been held out of contact with the emulsion by the fingers, is raised to a perpendicular position and attached to the topmost arm of the clamp. The attachment is accomplished by means of a small wire retainer made by bending both the small loop and the long end of the large loop of an ordinary paper clip down in the same direction until they are parallel to each other and perpendicular to the unbent, horizontal portion of wire. The wire of the small loop is then cut just above its lower curve. The ends of the two straight parallel wires of the cover slip retainer fit into small holes drilled through each end of the uppermost arm of the clamp. The cover slip is held between the clamp and the horizontal portion of the retainer.

The following is an enumeration of all the succeeding steps of the entire autoradiographic process:

1. Punch several small holes in one end of the cover slip. 2. Mount the bone sections on the other end of the cover slip. 3. Cut a rectangular section out of the center of the plastic cover slip. 4. Clamp the cover slip to the nuclear track plate and cement the end containing the small holes to the nuclear track plate with the special wax. 5. Remove the clamp. 6. Expose the track plate with a lead weight resting on that end of the cover slip on which the bone sections are mounted. 7. After the exposure, remove the lead weights and place the undeveloped autoradiographs in the plate carrier. 8. Fill the 3 compartments of the processing tank with developer, short stop, and acid fixer solutions respectively. 9. Process the plates. 10. Remove the processed autoradiographs from the plate carrier. 11. Prepare for washing by attaching the clamp and holding the cover slip perpendicular to the track plate with the cover slip retainer. 12. Wash the plates. 13. Remove clamp and cover slip retainer when the plates are dry.

The 3 requirements of a technique suitable for the production of autoradiographs to be used in the study of the distribution and concentration of a radioactive substance metabolically deposited in bone are fulfilled by this technique. (1) The attachment of the flexible cover slip containing the bone sections at one end with the special wax permits the bone sections to be removed from the portion of the nuclear track plate being processed. The bone sections are protected from contact with the processing solutions by the plate carrier. (2) The secure attachment of the cover slip to the nuclear track plate at one end also permits the bone sections to be removed from the nuclear track emulsion during the making of photomicrographs. (3) Since the cover slip is constantly attached to the nuclear track plate at one end, the bone sections mounted on the other end always return to exactly their original positions when desired.

The plate carrier, with a capacity for eight plates, and the motor-driven agitator prove to be valuable time-saving devices, for it is now possible to produce twice as many autoradiographs in a certain period of time as was formerly possible. This may be attributed to the capacity of the plate carriers and the actual reduction of the processing time, evidently due to superior agitation. Moreover, the quality of the autoradiographs is greatly improved, for no discoloration of the nuclear track plates is encountered.

Received October 23, 1950. P.S.E.B.M., 1950, v75.

Histopathology of the Liver Following Histamine Infusion.* (18330)

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One of the important aspects of hepatic physiology is the study of effects wrought by changes in the circulatory conditions in the liver. The present report is part of a series of such studies in this laboratory and is con-

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* This work was supported in part by a grant (to R.W.B.) from the Research Grants Division, U. S. Public Health Service.

cerned with histopathological changes during the continuous infusion of histamine into the portal vein of dogs. A more extensive discussion of physiological variables will be presented elsewhere(1).

Methods. Healthy mongrel dogs of either sex, kept 2 or more weeks in the laboratory on standard kennel ration, were used. Sodium pentobarbital anesthesia (33 mg/kg intravenously at the start and 5 mg/kg every 90 to 120 minutes if needed) was employed. Mean arterial blood pressures were recorded from the left carotid artery. For the continuous infusions a catheter was inserted at laparotomy into a mesenteric vein, and passed thence into the portal vein; the wound was closed by clips, and the free end of the catheter was connected to an infusion set consisting of Marioøtte flask, drop counter, and tunnel clamp. The solutions infused were 0.9% NaCl in the controls and 2.0 x 10^{-4} M histamine diphosphate in 0.9% NaCl in the experimental animals. Collections of blood and liver lymph were carried out as described elsewhere(2). Bromsulphthalein concentrations in the blood were determined by the method described elsewhere(3). Twenty mg of dye were used and the values reported as mean percent of dye removed from the plasma in 5 minute periods. Tissue blocks of the liver, for histological study, were removed prior to sacrificing the animals and were fixed immediately in 10% neutral formalin. In several of the experiments, tissues were taken from other organs for histological examination. Paraffin embedding, and hematoxylin and eosin staining were used.

Results. Infusion rates were gauged to maintain arterial blood pressures 45 to 55% of the preinfusion level. This required initial infusion rates from one to three cc/min. in various dogs; during the course of most experiments the rate of infusion had to be gradually accelerated up to 3 to 6 cc/min. Even after 4 or 5 hours of such infusion interruption of histamine flow invariably resulted in recovery of arterial blood pressures to preinfusion values within 15 to 25 minutes. Saline infused at rates comparable to the histamine infusions caused no significant changes in arterial blood pressure.

TABLE I. Circulatory Changes During Histamine or Saline Infusions Into the Portal Vein of Dogs.

No. of dogs*	Material infused	Blood pressure during infusion —% of initial	% hematocrit		% BSP—5 min.	
			Before infusion	During infusion	Before infusion	During infusion
8	Histamine sol.	60.9 ±6.4	49.1 ±4.2	48.4 ±4.0	59.6 ±3.5	47.8 ±4.2
7	Saline	92.2 ±7.8	42.8 ±4.8	42.9 ±4.4	65.3 ±3.8	56.4 ±6.5

* These dogs were selected at random for BSP studies before conducting an experiment.

TABLE II. Effects of Intraportal Histamine Infusion Upon Relative Liver Lymph Flow and Blood Pressure.

(1) Dog No.	(2) Arterial blood pressure—% of initial during hist. infus.	(3) Lymph flow g/min. during saline infusion	(4) g/min. during hist. infusion	(5) Ratio of 4 to 3
47-49	85	.037	1.069	35.0
48-13	53	.098	.349	23.6
49-3	85	.047	.594	12.6
49-4	33	.354	1.30	3.6
49-6	72	.020	.185	9.3
49-9	83	.065	.303	5.5
49-10	46	.051	.636	12.3
49-37	91	.067	.127	1.9

Table II shows the increase of liver lymph flow during histamine as compared to saline infusion. Liver lymph flow is considerably more sensitive to histamine infusion than the arterial blood pressure.

BSP extraction is slightly reduced by saline as well as by histamine infusions. A significant effect of histamine on this function was not observed. (Table I).

In the control group of 12 animals, which

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1. Brauer, R. W., and Nothacker, W. G., to be published.

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3. Brauer, R. W., and Root, M. A., *Am. J. Physiol.*, 1947, v149, 611

were subjected to 480 to 600 minutes of saline infusion, the only noteworthy gross change in the liver was prominence of the portal areas. Microscopically the only demonstrable change was dilatation and congestion of the portal lymphatics.

In the 19 experimental animals the livers became enlarged, boggy and mottled red and red-brown in color 3 to 5 minutes after beginning the histamine infusion, and remained so throughout the experiments. There were dilated subcapsular lymphatics in addition to prominent portal areas.

Histologically the livers from these animals may be divided into 2 groups. In Group I, 4 animals which died in shock or were sacrificed after 45 to 165 minutes of histamine infusion, changes were minimal and consisted only of constriction of the central veins, congestion of sinusoids and dilatation of portal lymphatics. In Group II, 15 animals infused for 220 to 540 minutes, the portal veins (Fig. 1) were dilated, congested and their walls were infiltrated with neutrophils and lymphocytes. The fibrous tissue stroma of the portal areas was edematous and infiltrated with variable numbers of neutrophils, lymphocytes, and eosinophils. An occasional portal area was diffusely infiltrated (Fig. 2) with leukocytes or contained a hemorrhage. The portal lymphatics were dilated and congested with lymph. The sinusoids were dilated and congested, and an occasional neutrophil was marginated. Hyaline droplets were occasionally present within the liver cells. The central veins (Fig. 3) were markedly constricted and bloodless. The surrounding stroma was congested, edematous and infiltrated with neutrophils, lymphocytes, and macrophages. Eosinophils were sparse.

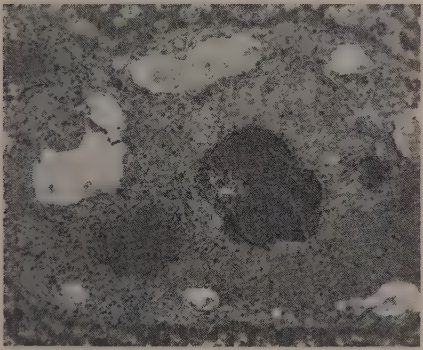

Fig. 1.

(Dog 47-70) portal area. Note the congested portal vein with the cellular reaction in the vessel wall which extends into the surrounding stroma. The dilated lymph channels are partly filled by coagulated lymph, and the edematous stroma contains scattered inflammatory cells. Hematoxylin and eosin ×60.

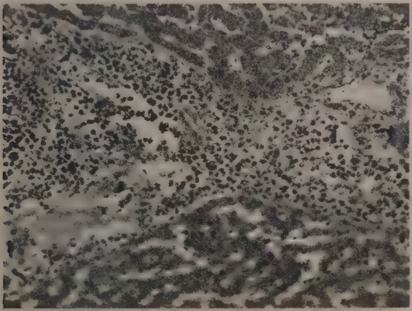

Fig. 2.

(Dog 49-15) portal area with diffuse inflammatory cell infiltration. Hematoxylin and eosin ×60.

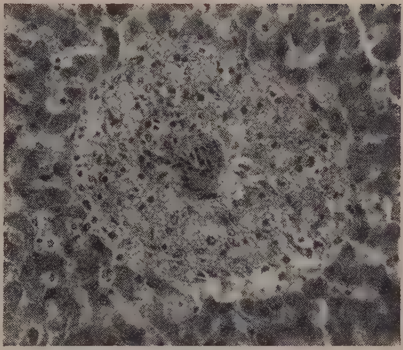

Fig. 3.

(Dog 47-70). The central vein is constricted and the surrounding stroma is edematous. Scattered inflammatory cells are present and the sinusoids are dilated and congested with red blood cells. Hematoxylin and eosin ×125.

Heart, lungs, spleen, pancreas, stomach, ileum, adrenals, and kidneys in several histamine treated animals were studied, in addition to the livers. However, no gross pathological or noteworthy microscopic changes were present in any of these specimens.

Discussion. Under the experimental conditions chosen irreversible shock does not set in even after 4 to 5 hours of histamine infusion; thus, low blood pressures must be maintained by increasing doses of histamine as each experiment progresses, and prompt recovery follows interruption of the infusion. Hematocrits remained constant throughout as a result of the infusion.

Barring marked changes in the arterio-hepatic venous gradient of BSP (which were not found)(1) the failure of histamine to significantly affect BSP clearance in these experiments indicates that total hepatic blood flow was little altered by the drug. Since there are indications of reduced portal venous blood flow (due to reflex vasoconstriction of splanchnic bed among other things) this observation suggests that the proportion of arterial blood supplied to the liver is increased under histamine infusion.

Hepatic lymph flow increases within a few minutes of the start of histamine infusion; it is preceded by visible engorgement of the liver. These observations suggest an increase of intrahepatic blood pressure, in accord with older reports(5) of a histamine activated sluice mechanism in the hepatic venules of the dog. To what extent increased permeability of the sinusoidal lining (similar to the "second permeability phase" described by Knisely)(6) also contributes to the increased lymph flow is uncertain at present. The cell content of hepatic lymph does not increase greatly unless arterial blood pressures fall below 20 to 25% of initial. These, in brief, are the circulatory conditions underlying the anatomical changes.

Histological evidence of liver cell damage is minimal (hyaline droplets in the cytoplasm of liver cells), and the early changes observed are confined almost exclusively to the vascular system. The late stage is one of exudation of leukocytes into portal and central vein regions in addition to the vascular changes. The leukocytic reaction is confined almost entirely to portal and central vein areas. The absence of central necrosis of the lobules in the presence of a leukocytic reaction differentiates the lesion described from that seen in pure chronic passive congestion(7). The histological picture described here differs also from that reported as a result of single large doses of histamine(8); the lesion under those conditions consists primarily of extensive extravasation of erythrocytes, and reflects conditions of irreversible shock with blood pressures of 20 to 30 mm Hg.

Summary. 1. The continuous infusion at sub-shock rates of histamine into the portal veins of dogs for periods exceeding 220 minutes results in the production of a definite liver lesion, distinguishable from the congestion observed after periods of histamine infusion less than 90 minutes, from the lesion of prolonged profound histamine shock, and from the cirrhosis of chronic histamine exposure. 2. The animals are not in shock and recover following discontinuance of the histamine infusion. 2. Liver lymph flow is greatly increased by histamine infusion, while bromsulphthalein extraction is reduced only slightly. Circulatory conditions in the histamine treated liver have been discussed briefly in terms of these findings. 4. In a series of control animals receiving prolonged saline infusions into their portal veins definite histological changes were not observed in the liver.

The authors are indebted to Mr. Antol Herskovitz, Montefiore Hospital, New York for the photomicrography.

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Received November 14, 1950. P.S.E.B.M., 1950, v75.

Uniform Transfer to Random Bred Rats of Lymphatic Leukemia Induced by Gastric Instillation of Methylcholanthrene. (18331)

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We have recently reported(1) the development of lymphatic leukemia in various members of our random bred colony of Wistar rats following the gastric instillation of methylcholanthrene. Initial efforts to transmit the leukemia to a group of rats, 32 to 44 days of age, met with only indifferent success. Recently Gross(2) reported the transmission of lymphatic leukemia from Ak mice to C_3H subline by using animals 1 to 7 days old. By employing rats in this age group, we not only obtained a uniform transfer to animals of our own colony, but to rats obtained from the current Wistar Institute colony and to random bred rats of the Sherman and Long-Evans strains. All of the transfers described in this report were accomplished by the intraperitoneal injection of a suspension of leukemic spleen cells.

Procedure. The spleen from a leukemic animal was put through a special masher* and then mixed thoroughly with an approximately equal volume of normal saline solution. Injections of 0.2 cc of this cell suspension per animal were used. Table I contains the total number of transfers made and thus far represents transmission of the disease through 14 generations. It also lists the groups of animals older than 7 days to which transfer of the leukemia was attempted. Similar to the results of Gross in mice, we found a sharp decline in the number of successful transfers when animals past the age of 15 days were used.

TABLE I. Lymphatic Leukemia.

Age of animals at transfer, days	No. of animals	No. developing lymphatic leukemia
1-7	161	161
15	27	24
30	33	18
40	27	7

Results. Among 18 rats from 32 to 44 days of age injected intraperitoneally with suspensions of spleen cells from animals which had developed leukemia after the gastric instillation of methylcholanthrene, only 3 developed leukemia. These were diagnosed 19, 29, and 30 days after injection and at the time of sacrifice the white counts ranged from 47,000 to 340,000 with 31% to 95% blast forms. Histologically, the tissues were widely infiltrated with leukemic cells. Of the 18 animals in this group those under 35 days of age are included in the 30-day-old group in Table I, while those older than 35 days are included in the 40-day-old group. In contrast, 100% success was achieved with the 161 rats from 3 to 7 days of age injected in a similar manner. These animals all showed peripheral blood evidence of lymphatic leukemia within 8 to 18 days after inoculation and if not sacrificed died usually within 25 days of injection.

Uniform transfer of the disease was also achieved in 2 litters totaling 9 rats born to females obtained from the current random bred Wistar colony of the Wistar Institute from which the original animals for the development of our colony were obtained more than 12 years ago, as well as in 13 rats of the Sherman strain and 18 of the Long-Evans strain born to mothers obtained from random bred colonies of these strains (Rockland Farms). The ages of these rats at the time of transfer were 3 to 7 days and all developed leukemia as readily as did the young rats of our own colony.

Comment. The uniform transfer of the lymphatic leukemia, developed through the gastric instillation of methylcholanthrene, to very young animals of 3 different strains of rats and only a limited transfer to 40-day-old animals, suggests the presence of some

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2. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 246.

* We are indebted for the detailed design of this instrument to Ernest Sturm of the Rockefeller Institute.

mechanism that resists invasion by foreign cells, which appears to be absent at birth and for a short time thereafter, but which then develops rapidly. The possibility of such a mechanism appears in the studies of Duran-Reynals(3) with birds, those of Gross with mice and in our own with rats.

Summary. Lymphatic leukemia induced in Wistar rats by the gastric instillation of methylcholanthrene, was transferred to 40-day-old animals of our random bred Wistar colony with only indifferent success. When transfers were made to animals not older than 7 days, these were successful in 100% of the animals. Transfer of the leukemia was also made uniformly to equally young animals of the Sherman and Long-Evans strains. The intraperitoneal injection of leukemic spleen cells was used in these experiments.

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Received August 18, 1950. P.S.E.B.M., 1950, v75.

Effect of Crystalline Vitamin B_{12} on Experimental Anemia in Mice.* (18332)

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Crystalline vitamin B_{12} has been shown to produce favorable hematological response and general clinical improvement in cases of Addisonian pernicious anemia. It has been reported(1) that vit. B_{12}, when effective, is active in smaller doses than folic acid or thymine but the interrelations between these antianemic substances have not been clearly established. The potency of vit. B_{12} preparations has been determined on the basis of the clinical response of pernicious anemia patients(2,3) and assays have been made based on the growth of microorganisms (*Lactobacillus leichmannii* and *Lactobacillus lactis* Dorner)(4-8).

The experiments reported here were performed to determine the effect of vit. B_{12} on the regeneration of red blood cells and hemoglobin in mice made anemic by the intraperitoneal injection of phenylhydrazine. Phenylhydrazine has been employed as a mild hemolytic agent in *Polycythemia vera*(9). According to Long(10) the mature circulating red cells of laboratory animals and humans are hemolyzed but the erythroblastic elements of the bone marrow are not injured by this drug. In other studies on the hemopoietic activity of proteins and amino acids phenylhydrazine was used to induce severe hemolytic anemia in rats and rabbits(11,12). Damodaran and Vijayaraghavan(13) observed that liver extract (Cohn's fraction G) caused a marked increase in erythrocytes, but not hemoglobin, of rats rendered severely anemic with phenylhydrazine. More recently Nichol *et al.*(14) reported that neither liver extract nor vit. B_{12} stimulated hemoglobin regeneration in chicks which were depleted of folic acid and made anemic with phenylhydrazine. A stimulatory effect was noted when folic acid was administered.

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* Paper 77. This work was aided by grants from the Beech-Nut Company, the Nutrition Foundation and the University of California. The authors are indebted to Mr. E. A. Murphy for valuable suggestions and to Merck Institute for Therapeutic Research through the courtesy of Dr. Gladys A. Emerson for a sample of crystalline vit. B_{12}.

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Experimental. The basal diet was of the following composition: Vitamin test casein (General Biochemicals' product) 15, sucrose 71, lard (Swiftning) 8, and salt mixture 6 (15). The mg of vitamin supplements added per 100 g of diet were thiamine .5, riboflavin 1, pyridoxine .5, niacin 10, *p*-aminobenzoic acid 20, inositol 100, folic acid .02, biotin .006, ascorbic acid 40, calcium pantothenate 2, choline chloride 50, vit. K (Menadione) .1, alpha tocopherol 5, vit. A 2000 (U.S.P. units) and vit. D 200 (U.S.P. units). It is probable that vit. B_{12} was present, but at a relatively low level, in this diet.

Adult mice from A strain parents were conditioned by maintaining them on the basal diet for 3 days prior to the injection of phenylhydrazine hydrochloride. On the following (fourth) day the tail was clipped and blood was collected discarding the first 1 or 2 drops. Red blood cell counts were made on blood samples diluted 1:200 using Hayem's fluid and Spencer's bright line counting chamber. Hemoglobin was determined by the alkaline oxyhemoglobin method(16) using a photoelectric colorimeter (Lumetron, Model 400-A).

Anemia was produced by a single intraperitoneal injection of .5% aqueous solution of phenylhydrazine hydrochloride in the dosage (6 to 8 mg per 100 g of body weight) required to reduce the red blood cell count and the hemoglobin concentration to 50 and 60%, respectively, of their normal values.‡ The comparable dosage for rats is 2 to 3 mg. In the first experiment on red blood cell and hemoglobin regeneration 3 μg of vit. B_{12} (Squibb's crystalline Rubramin) were injected (intraperitoneally) daily for 10 days into each anemic mouse (6 control and 7 experimental). In the second experiment 1 to 4 μg of vit. B_{12} (Merck's crystalline product) were injected (intraperitoneally) daily for 4 days into each anemic mouse (5 control and, at each level of vit. B_{12}, 6 experimental).

Discussion. Food consumption averaged 3 to 5 g daily per mouse during the first 4 days following the injection of phenylhydrazine hydrochloride and 4 to 6 g thereafter. There was no significant difference in body weight or food consumption between the control and the test animals during the experimental period.

As shown in Fig. 1 the normal red blood cell count of the control animals was regained on the eighth day and of the vit. B_{12}-treated group on the fourth day after the peak of

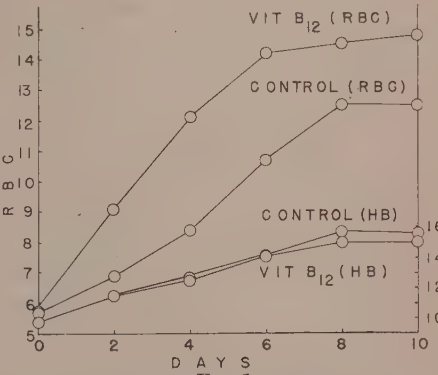

FIG. 1.

Rate of regeneration of red blood cells and hemoglobin in mice with and without vit. B_{12}. R.B.C.—Red blood cells expressed as millions per cu. mm. H.B.—Hemoglobin expressed as g per 100 ml. The values plotted are from the 4th day after injection of phenylhydrazine hydrochloride.

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‡ The normal red blood cell count of the mouse was found to average 12.4 (12.0-12.7) millions per cu mm compared to 7.5-8.0 for the rat. The hemoglobin concentration (14.5-15.5 g per 100 ml) is the same for both animals.

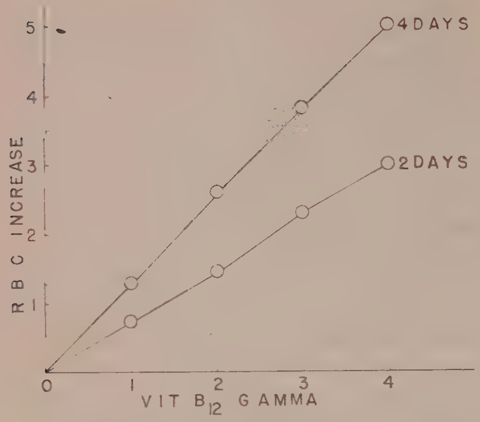

FIG. 2.

Increase in R.B.C. (red blood cells) over control at different levels of vit. B_{12}. R.B.C. count expressed as millions per cu. mm. Each value represents the average from a group of 6 animals.

the anemia. It is of interest that on the sixth day the red blood cell count of the test animals increased to values higher than normal. As indicated the concentration of hemoglobin was not influenced by the administration of vit. B_{12}. Presumably the size of the erythrocytes newly formed under the influence of vitamin B_{12}, the hemoglobin content of the cells, the total blood volume and possibly other factors account for these findings which are in striking contrast to the effects of vit. B_{12} noted in pernicious anemia as well as other types of megaloblastic anemias.

The increases in numbers of red blood cells observed in the test animals at each of the 4 levels of vit. B_{12} for periods of 2 and 4 days are shown in Fig. 2. At 4 days particularly it may be noted that the increase in red cell counts was directly proportional to the concentration of vit. B_{12} administered. These results suggest that an assay procedure for the determination of vit. B_{12} could be developed in terms of the increase in red blood cell counts at different dosages of vit. B_{12}. Experiments toward this end are in progress.

Summary. The number of red blood cells per unit volume, but not hemoglobin concentration, was increased by crystalline vit. B_{12} injected intraperitoneally into mice rendered anemic by the administration of phenylhydrazine hydrochloride. The mice were maintained prior to and during the experiment on a chemically-defined diet probably containing a relatively small amount of vit. B_{12} but adequate quantities of other hematopoietic substances. The increase in red blood cells, particularly during the first 4 days of severe anemia, was found to be directly proportional to the amount of vit. B_{12} administered. These results suggested the possible development of an assay procedure for the determination of vit. B_{12}.

Received August 21, 1950. P.S.E.B.M., 1950, v75.

Some Spectrophotometric Observations on Invertebrate Nerves and their Extracts.* (18333)

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Recent experimental results have led to the suggestion that metabolic reactions may be intimately associated with the subliminal and threshold response of nerve. Optical methods, such as employed by Keilin(1), Urban and Peugnet(2) and Arvanitaki and Chalazonitis(3), appear to offer the means of fol-

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* Supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

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lowing such reactions at rates comparable to those of the bioelectrical phenomena.

In the present study the possibility of detecting specific enzyme systems spectrophotometrically has been explored for intact nerves of *Libinia emarginata* and *Loligo pealii.* Observations have also been made on extracts from these nerves both in visible and ultraviolet light.

Methods. The leg nerves of Libinia, obtained by the method of Shanes and Hopkins(4), and the fin nerves of Loligo were employed. All nerves were ligated at both ends and suspended in sea water for 5 to 15 minutes before being mounted. They were then placed in standard, 10 mm corex glass cells, where they were maintained in position by the gentle pressure of a perforated metal plate. Absorption data were obtained with the Beckman spectrophotometer. A modification was made which consisted of the insertion of brass tubes into one side wall of the cell compartment. These tubes were connected by rubber tubing to a specially designed metal cap which fitted snugly over the experimental absorption cell. A metal tube extended from the cap to about 2 mm from the bottom of the glass cell. Humidified oxygen or specially purified "Linde" (99.9%) nitrogen entered by way of this tube and left by way of an exit tube at the top of the cap. During the course of the experiments a continual, gentle bubbling of gas was maintained through the cell. The control cell carried an identical perforated plate and contained the same medium. The calibration of the spectrophotometer was checked for visible light with the major sodium and potassium emission lines and for the ultraviolet with desoxyribonucleic acid. A typical experimental series consisted of 3 sets of measurements of the nerve absorption over the wavelength range of 1000 to 400 mμ, the first and third set being in oxygenated, the second in nitrogenated sea water. After the completion of each set of determinations the contents of the experimental cells were usually decanted and stored in the refrigerator at 4°C overnight for examination the following day. At the end of the series the nerves were placed into 5 cc of distilled water and refrigerated. A number of aqueous extracts were prepared with macerated nerves placed into 3 to 5 cc of distilled water, for periods of time varying from 30 minutes to 24 hours. The longer extractions were carried out at 4°C. The extracts and the sea water which had been in contact with the nerves were also examined in ultraviolet light over the wavelength range of 300 to 240 mμ. The dialysis of the nerve extracts was performed by placing 10 cc of extract into a dialyzing bag, in turn suspended in a beaker containing 10 cc of distilled water and mounted on a shaker table. Samples were taken after one hour and 4 hours both from the bag and from the dialyzate. After 4 hours the bag was kept in running water, the residue being tested after 24 and 48 hours. For comparative purposes some observations have also been made on Loligo axoplasm, Libinia muscle extracts, and Libinia blood. All experiments were performed at room temperature—approximately 20° to 24°C. It was noted that the temperature of the contents of the cells increased approximately 4°C during the course of the determinations lasting 25 to 50 minutes.

Results. Visible light. As may be seen in Fig. 1, a series of peaks in intact nerves lies between 568 and 538 mμ. They are more clearly defined in the absence of oxygen and occur within a range of ± 2 mμ at 542, 550 and 556 mμ. Sodium hydrosulfite accentuates these peaks to a somewhat more marked extent than nitrogen under the conditions employed, and makes an additional peak evident at 568 mμ. Some recovery was noted in the hydrosulfite treated nerves when they were washed in sea water or oxygenated. It seems likely that the 550 mμ peak is that of cytochrome c. Further experiments are necessary, however, for the positive identification of this and the other peaks observed. Absorption curves obtained for one sample of Loligo axoplasm demonstrated similar peaks only in nitrogen. Examination of the aqueous

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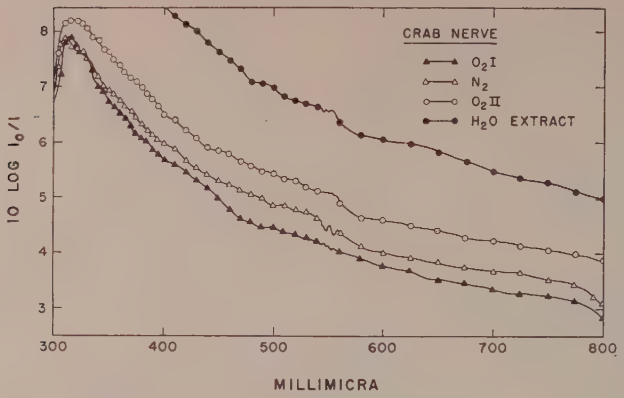

FIG. 1.

Absorption curves for one Libinia nerve subjected to oxygen, then nitrogen, and finally oxygen again, and for its 16 hr aqueous extract. The apparent peak below 400 mμ is an instrumental artifact.

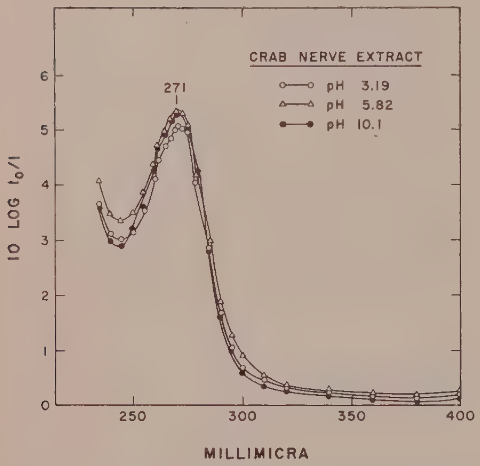

FIG. 2.

Typical absorption curves obtained with the same nerve extract at the indicated pH values. 7.5 normal acid and 2.5 normal alkali had no greater effect on the peak.

extracts and of the sea water which had been in contact with the nerves indicated that the peaks were present to a slight degree.

Ultraviolet light. Sea water which had been in contact with the nerves, as well as the aqueous extracts, gave a single, sharp peak which consistently occurred within one mμ of 271 mμ (Fig. 2). Fifteen cc distilled water, after one hour contact with 100 mg nerve tissue, gave a maximum peak density of approximately 0.5 in the standard 10 mm quartz cell. This peak was insensitive to pH changes ranging from 3.2 to 10.1. The same absorption maximum was obtained in Loligo fin nerve extracts and for the protoplasm of the giant axon of Loligo. The common occurence of this peak suggested the examination of other tissues. It was found that Libinia muscle extracts and Libinia blood gave distinctly different maxima; the former, which is little altered by pH, gave a maximum at 267 mμ, and the latter at 278 mμ.

The nature of the substance responsible for the 271 mμ peak can not be specified as yet. It is not a protein since it gives a negative Biuret test, is not precipitated by perchloric acid, and is readily dialyzable. It is highly polar, for it is not extractable from aqueous solution by ether or amyl alcohol.

The dialysis experiments demonstrated the complete removal of the unknown in 24 hours. After this period the residue had a peak between 265 and 268 mμ, while after 48 hours the peak was found to have shifted to 275 mμ.

Summary. 1) A method is presented for the spectrophotometric observation of intact, invertebrate nerves and their extracts in the presence or absence of oxygen. 2) Absorption maxima were observed for intact nerves between 538 and 568 mμ, and at 271 mμ for their aqueous extracts.

The authors are indebted to Dr. M. A. Jakus and Dr. A. J. Hodge of the Massachusetts Institute of Technology for the Loligo axoplasm; to Mr. W. S. Vincent of the University of Pennsylvania for the DNA; to Dr. Alan Mehler of the University of Chicago for his many helpful suggestions, and to Mr. Olivier Heroux of Laval University, Quebec City, P. Q., Canada, for his generous assistance, particularly in the dialysis experiments.

Received October 9, 1950. P.S.E.B.M., 1950, v75.

Concentration of Streptomycin in Brain and Other Tissues of Cats After Acute and Chronic Intoxication.* (18334)

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The disturbance of equilibrium which can be produced in the cat by the parenteral administration of suitably large doses of streptomycin develops only after several days of treatment(1). The time of onset of the characteristic ataxia, accompanied by loss of nystagmus in response to rotation, varies inversely with the size of the dose employed (Fig. 1). The manner in which this cumulative toxic effect is produced is still unknown. The experiments to be described were made to test one possible explanation, namely that chronic streptomycin intoxication is associated with an accumulation of the drug in the cerebrospinal fluid and in the central nervous system. The negative results, demonstrating that such an accumulation does not occur, are in accord with histological(2,3) and electrophysiological(4,5) studies which have been published since this work was done, indicating that the primary site of the toxic action of streptomycin is not in the brain stem but rather in the vestibular and cochlear end-organs. On the other hand the present experiments are of interest because they show that, unlike the brain, other organs such as the lungs and liver contain significant concentrations of the drug even 72 hours after a single dose.

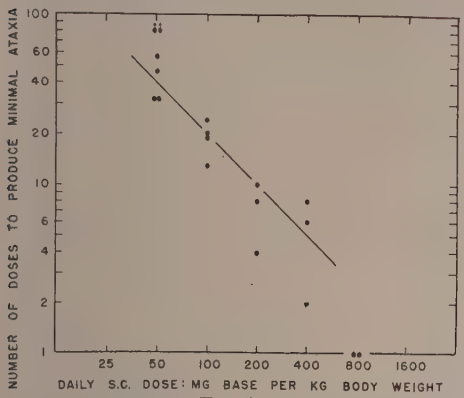

Fig. 1.

No. of doses of streptomycin required to produce minimal ataxia in cats, plotted as a function of the size of the daily dose. The straight line represents a *constant total dose* of 2 g per kg, *e.g.*, 200 mg/kg/day for 10 days. The data may be interpreted to mean that within the range 50 to 400 mg/kg an approximately constant total quantity of streptomycin is required to produce signs of vestibular disturbance. A single dose of 800 mg/kg is lethal. Streptomycin hydrochloride Merck, Lot No. 731. (From data of Hawkins and O'Shanny presented at the meeting of the Fed. Am. Soc. Exp. Biol., Atlantic City, March 1948).

Method. The fluorometric method for the determination of streptomycin in body fluids(6) and in tissues(7) was employed.

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TABLE I. Acute Intoxication. Streptomycin concentrations in body fluids and tissues of cats after single dose of 400 mg per kg S.C.

Cat	Hr after dose	Serum	CSF	Hemispheres	Cerebellum	Brain-stem	Lung	Liver
1	2	796	5					
	5	680						
	24	116	35	14	18.5	14	86	84
2	2	517	5					
	5	313						
	26	21	1.5					
	48	1.5	1	2.5	3.5	4	17	20
3	2	874						
	5.5	464						
	26.5	31						
	48.5	4						
	72	1	2	2	4	3.5	23	38

SM conc. in body fluids, μg per ml; in tissues, μg per ml (wet wt).

The lower limit of sensitivity of the method for body fluids is 1 μg of streptomycin in 1 ml of fluid, and for tissues 2 μg per g of tissue.

Acute Intoxication. In order to determine whether streptomycin remains in the brain after a single large dose, the drug in the form of the calcium chloride complex was given to 3 cats by subcutaneous injection of a dose equivalent to 400 mg streptomycin base per kg of body weight (corresponding to 24 g for a 60 kg man). Typical signs of acute intoxication appeared: vomiting, hyperpnea, prostration and finally stupor lasting for several hours. Two hours after the injection the concentration of the drug in the serum ranged from 500 to 900 μg per ml. No tissues were taken for analysis at this time, however, since the actual concentration in the tissues cannot be determined in the presence of high levels in the blood. Instead the method chosen was to measure the concentration remaining in the tissues at a time when it was expected that removal of the drug from the blood by renal excretion would be almost complete. The 3 animals were sacrificed and tissues were taken for analysis only after 24, 48 and 72 hours respectively. Data from this experiment are presented in Table I. It was concluded that streptomycin does enter the brain after a very large dose and can persist there for as long as 3 days. The amount in the brain, however, is small as compared with that in other tissues.

Chronic Intoxication. For repeated subcutaneous administration a dose of 100 mg streptomycin base per kg per day was chosen. Four cats were so treated for 7, 14, 21 and 28 days respectively. The serum concentrations of streptomycin obtained after the first dose, which was given intramuscularly, are shown in Fig. 2. One representative case is illustrated, since the curves for the 4 cats are practically superimposable. Blood samples taken on various days during the experiment, in each case 24 hours after the previous injection, showed plasma concentrations of 1 to 4 μg per ml. There was no evidence of accumulation of streptomycin in the blood or CSF with continued treatment, a finding which is in agreement with the experiments on the effect of multiple doses on blood concentrations reported by Boxer *et al.*(8). Data obtained at the time of sacrifice of these four animals are presented in Table II.

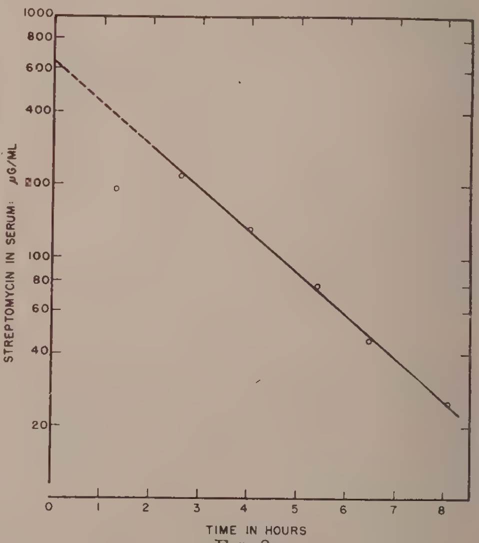

FIG. 2.

Streptomycin concentrations in the serum of cat 6 after a single intramuscular inj. of 100 mg base/kg.

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TABLE II. Chronic Intoxication. Streptomycin concentrations in body fluids and tissues of cats after daily doses of 100 mg per kg SC.

Cat	No. of doses	Hr after last dose	Ataxia	Serum	CSF	Hemispheres	Cerebellum	Brain-stem	Spinal cord
4	7	48	—	1	<1				
5	14	24	1+	2	1	1	1	1	—
6	21	24	4+	3	2	2	2		1
7	28	24	4+	2.5	3	1	1	1	1

Cat	Lung	Liver	Spleen	Parotid	Kidney	Urine
4	7	16.5	12	7	119	21
5	16	23	24	22	234	—
6	32	41	21	18	176	360
7	20	10	22	12.5	148	90

SM conc. in body fluids, μg per ml; in tissues, μg per ml (wet wt).

Discussion. The present experiments show that although a small quantity of streptomycin can be detected in brain tissue after a single large subcutaneous dose, there is no accumulation of the drug in the brain as a whole or in the brain stem during the development of a severe chronic intoxication. If there had been a striking accumulation in particular medullary nuclei, this should have been reflected in the concentration measured in the brain stem. On the contrary, there was no difference between the concentration in the stem and that in other portions of the brain. This finding is in agreement with the results of the investigations already cited(2-5), which demonstrate that the labyrinthine and cochlear end-organs are the primary site of the neurotoxic action. Unfortunately no data on streptomycin concentrations in the endolymph and perilymph have been obtained because of obvious technical difficulties. Nevertheless it is reasonable to assume that the concentration in the perilymph corresponds to that in the CSF, and that the drug reaches the end-organs by this route. The same considerations presumably apply in the case of the less neurotoxic dihydrostreptomycin(9,5).

Concentrations determined in the lungs, liver and other organs must represent streptomycin actually contained in the cells of these tissues and not merely in the extracellular fluid, where the concentration should correspond approximately to that in the serum. No conclusion can be drawn concerning possible binding to the tissue proteins, since the deproteinization technique employed in the analytical procedure liberates any protein-bound streptomycin.

The data in Table I indicate that streptomycin once present in the tissues is removed only slowly. When the values at 24, 48 and 72 hours after injection are compared, it is apparent that the fraction of the drug present in the blood and extracellular fluid is removed rather rapidly by excretion through the kidney, while the tissue concentration is decreasing at a much slower rate. A strikingly high tissue concentration was found in the kidney itself (Table II). This observation is in accord with that of Adcock and Hettig(10), who reported streptomycin in the kidneys of 2 patients who had died during treatment for tuberculous meningitis.

Summary. Streptomycin was found in the brain, lungs and liver of cats for as long as 3 days after a single injection of 400 mg per kg. At this time the drug had virtually disappeared from the blood and CSF. The concentration in the brain was small compared with that in the other organs. In cats showing typical chronic intoxication during treatment with streptomycin in daily doses of 100 mg per kg, no accumulation of the drug was found in the serum, CSF or brain 24 hours after last dose. Significant concentrations of the drug were present in other tissues, especially in the kidney. It is concluded that the chronic neurotoxic action of streptomycin cannot be attributed to accumulation of the drug in the brain as a whole or in the brain stem.

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Received October 13, 1950. P.S.E.B.M., 1950, v75.

Anemia Associated with Blind Intestinal Segments and its Prevention with Aureomycin.* (18335)

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This study was prompted by the observation of ulcerative lesions in blind intestinal segments resected from patients suffering from chronic anemia. Following the construction of a side to side intestinal anastomosis the blind end of the proximal segment is continually subjected to the driving force of intestinal peristalsis. In some instances this blind segment becomes markedly dilated and packed with fecal material. Black and McEachern(3) describe the usual symptoms as abdominal pain, cramps, diarrhea and vomiting. They reported 5 cases, 2 with ulceration and perforation and one with anemia. Pickhardt(11) and Eckel and Holman(6) reported symptoms including anemia occurring as late as 9 and 10 years after construction of a side to side anastomosis. Cameron *et al.*(5) described a case of macrocytic anemia which developed within one year after a side to side anastomosis in the terminal ileum for regional enteritis. Estes and Holm(7) reported 2 cases showing marked ulceration in such redundant blind intestinal segments and were able to reproduce this syndrome in dogs. Pearse(10) however found that ulceration and perforation did not occur in such dogs unless a foreign body was present in the intestinal lumen.

Our purpose was to ascertain whether or not such ulcerative lesions were a constant finding in blind intestinal segments created in the experimental animal.

Method. Adult male and female rats of the Sprague-Dawley strain weighing from 300-400 grams were prepared in a manner similar to that described by Watson, Cameron, and Witts(14). Under ether anesthesia the abdomen was opened and the small intestine divided about 5 inches distal to the ligament of Treitz. The cut end of the proximal segment was inverted and at a point 3 inches proximal to this inversion an end to side anastomosis was done with the distal segment using interrupted sutures of very fine silk. In this manner a blind intestinal pouch 3 inches in length was so constructed that intestinal peristalsis would tend to keep the pouch filled. The animals so prepared were divided into 2 groups. Series A consisted of 18 rats which were fasted for 48 hours after surgery and then placed on a standard laboratory diet of Purina foxchow biscuits. Series B of 5 rats was identical except that 50 mg of aureomycin were added to the daily portion of ground biscuit. A control animal of similar size and weight was kept in the same cage and on the same diet as each animal of series A and B. At weekly intervals all animals were weighed, a benzidine test of fresh feces done, and blood was obtained from the cut end of the tail for hematological examination. In addition to routine erythrocyte and leucocyte counts, hemoglobin was determined by the oxyhemoglobin method of Evelyn, and hematocrit by a modification of the technic described by Rosahn(13). When the hemoglobin level had dropped to 10 g or less the animal was sacri-

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* The researches presented here were supported by the following funds for research: 1. The Malignant Disease Fund for Surgical Research; 2. Augustus L. Searle Fund for Surgical Research; 3. Austen S. Cargill Fund for Surgical Research; 4. Graduate School of the University of Minn.

3. Black, B. M., and McEachern, S. G. O., 1948, v86, 177.

5. Cameron, D. G., Watson, G. M., and Witts, L. J., *Blood,* 1949, v4, 793.

6. Eckel, J. H., and Holman, C., *Surgery,* 1944, v15, 307.

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10. Pearse, H. E.. *S.G.O.,* 1934, v59, 726.

11. Pickhardt, O. C., *Ann. Surg.,* 1933, v97, 116.

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ficed. Post-mortem autolysis of the intestinal mucosa in a rat is very rapid. To circumvent this factor the animal was anesthetized with ether and the entire gastrointestinal tract filled with 4% formalin by means of a syringe and fine gauge needle. The abdominal viscera were then removed en masse and fixed in formalin.

Results. Anemia. From 104 examinations on normal control animals the following average values were determined: Hemoglobin, 15.4 g percent: erythrocyte count, 8.6 million: and hematocrit, 46.5%. On the basis of these normal values the color and volume indices were calculated according to Osgood(9).

The hemoglobin level fell to 10 g percent or less in 100% of the untreated rats in series A. The anemia appeared rather abruptly from 22 to 86 days after the formation of the blind segment and was accompanied by a weight loss of from 28 to 103 g. Characteristically, the animals appeared well for one to 2 months then suddenly became anemic and obviously quite ill. If the animal was not sacrificed, the anemia was invariably fatal, usually in 2 to 3 weeks after its first appearance. Color indices indicated a predominantly normochromic type of anemia. In those animals in which the hematocrit was determined, the volume indices showed a fairly even distribution between macrocytic and normocytic types of anemia.

None of the animals receiving aureomycin daily in Series B developed anemia in a period of 127 to 175 days after surgery. All remained well until sacrificed and showed a weight gain varying from 17 to 65 g.

None of the control animals developed anemia.

Melena. The presence of blood in the feces as determined by the benzidine test was never detected in the control animals. In Series A, melena was found very occasionally at weekly intervals in some animals and in others, not at all. When daily examinations were done over a week's time, the results were the same. In Series B, melena appeared only twice, both times shortly after operation and did not recur.

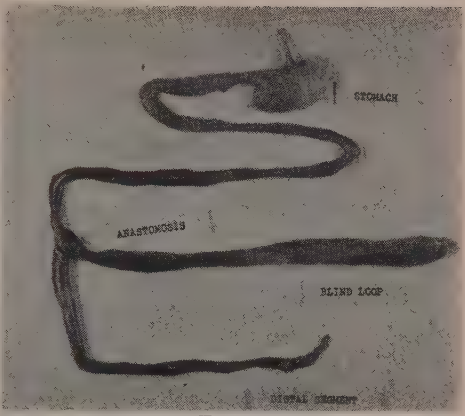

FIG. 1.

The blind intestinal pouch as constructed. Note relative size and position in the intestinal tract.

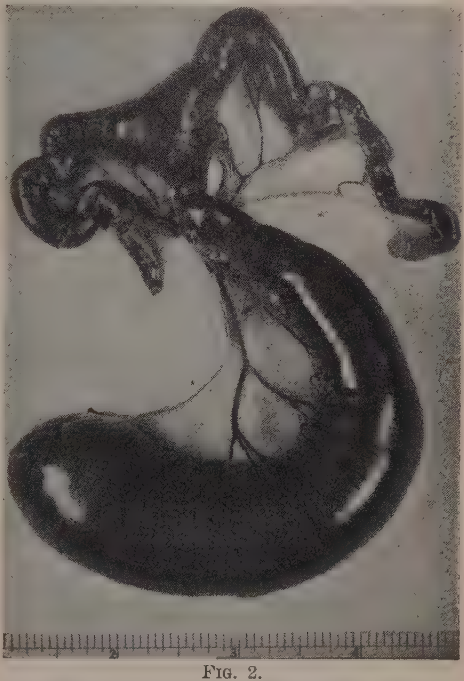

FIG. 2.

A typical example of the blind loop at the time of sacrifice. Note marked enlargement of loop. The efferent channel extends to the right.

Pathology. With the exception of 2 instances of parasites in the liver, all sections of that organ and of the spleen appeared normal. The

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9. Osgood, E. E., *A Testbook of Laboratory Diagnosis,* The Blakiston Co., Philadelphia, 1940.

most striking change that occurred in the blind intestinal segments was a marked increase in all dimensions (Fig. 1 and 2). Some segments were almost double their original length and the increase in diameter was even greater. All specimens appeared more vascular than normal. The majority of the blind segments were filled with very thin intestinal contents indistinguishable grossly from that in the remainder of the small intestine. In one animal gross examination revealed a superficial erosion in the distal part of the blind segment. True ulceration and perforation did not occur. Microscopically there was marked increase in the thickness of the muscularis in all cases, and most instances showed some increase in depth of the mucosal layer when compared to sections from above and below the site of anastomosis (Fig. 3 and 4). Two specimens showed evidence of vascular engorgement and hemorrhage into the mucosa. No ulcerations were found in 20 serially arranged sections from each blind pouch

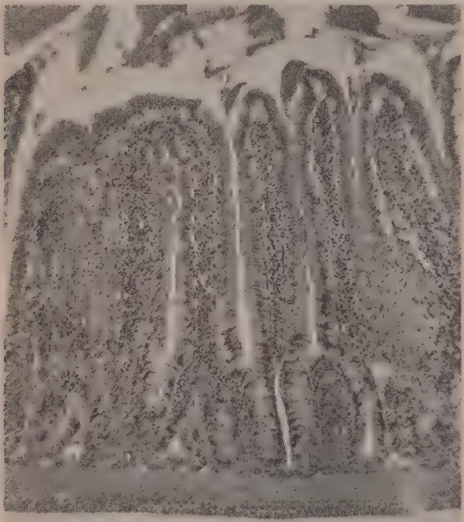

FIG. 3.
Microscopic section of normal rat's intestine. (×100).

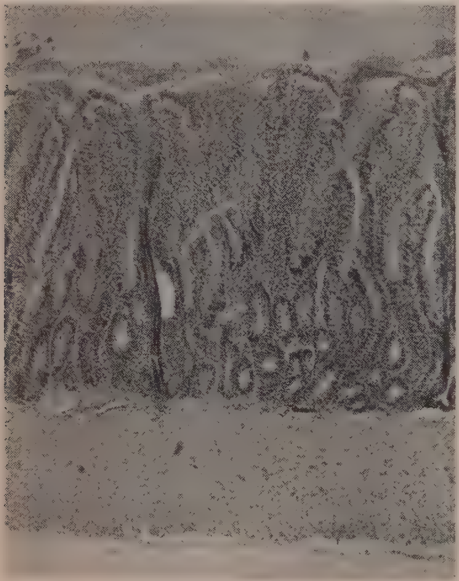

FIG. 4.
Microscopic section of intestinal wall from specimen shown in Fig. 2. Note increased thickness of muscularis.

Comment. It is apparent that some relationship exists in the rat between anemia and blind intestinal segments. In 100% of the animals in which such a segment was made and no therapy given, anemia eventually developed. It did not occur in any of the controls. The exact mechanism, however, by which such anemia is produced is not so definite. Alt(1) has shown that 40% of the rat's small intestine may be resected with no chronic ill effects. It is unlikely therefore that the loss of such a short segment as 3 inches would seriously interfere with the absorptive mechanism. Liquid stools were never observed in any of the animals. Although both operated and control animals in Series A were fed the same diet, some element of deficiency may be a factor in the later stages when the animals begin to lose weight.

It is difficult to indict chronic blood loss as a major etiological factor in the anemia on several scores. Ulcerative or erosive lesions were not demonstrable in the blind pouches of rats. This is in contrast to the clinical cases where erosions or ulcerations were consistently found in the distended terminal end of the proximal segment of a side to side anastomosis. The detection of blood in the stools was a very inconstant finding

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1. Alt, H. L., *Am. J. Dig. Dis. and Nutrition,* 1936-37, v3, 167.

TABLE I. Terminal Values.

Series	A			B		
No. of animals	18			5		
	Min.	Max.	Avg	Min.	Max.	Avg
Survival time, days	22	86	46.5	125	175	165
Weight change, g	—28	—113	—83	+17	+65	+45
Hemoglobin, g %	2.6	10.0	7.4	15.0	18.1	16.6
Erythrocytes, millions/cu mm	1.6	6.3	4.5	8.0	10.5	9.0
Leucocytes, thousands/cu mm	8.5	40.0	21.2	11.0	26.3	19.7
Hematocrit, %	12	36	26	47	57	51
Color index	0.73	1.02	0.92	0.86	1.15	1.04
Volume index	0.89	1.62	1.20	0.9	1.19	1.05

and the hypochromic type of anemia usually associated with chronic blood loss never occurred.

Intestinal stasis has been postulated by many authors as a prime factor in the etiology of this type of anemia. Butt and Watkins(4) reported 20 cases of anemia associated with such lesions of the bowel as terminal ileitis, ileal ulcer, tuberculosis with stricture, fecal fistula, etc. Of these anemias 11 were macrocytic, 5 microcytic, and 4 normocytic. They noted that the only cases of small intestinal ulceration that showed a macrocytic type of anemia also had obstruction. They suggested that any lesion of the bowel that produces obstruction may so interfere with the absorption or utilization of the hematopoietic principle that an anemia, usually macrocytic, will develop. Barker and Hummel(2) have reviewed 51 cases of macrocytic anemia associated with intestinal strictures and anastomoses. They subscribed to the hypothesis that the primary cause of the anemia was the absorption from the stagnant obstructed gut of a toxic material that interfered with blood formation. Little *et al.*(8), and Richardson(12) have reported on the occurrence of anemia, usually macrocytic in type, following short circuiting operations for small intestinal obstruction secondary to inflammatory adhesions. In these cases the by-passed intestine constituted a blind segment and an element of intestinal stasis. Cameron, *et al.*(5) described a similar situation in a case of regional enteritis and suggested toxic absorption as the most likely etiology.

Although by no means conclusive, the evidence in these animals would favor the thesis of intestinal stasis and unusual bacterial activity as a significant factor in the etiology of the anemia. In all animals a marked enlargement and hypertrophy of the blind segment characteristic of intestinal obstruction was observed and the majority showed filling of these segments with fecal material to the point of distention. In addition none of the operated animals that were fed aureomycin—a known antibiotic—developed anemia during a similar time interval.

Conclusions. 1. Anemia, either macrocytic or normocytic in type, can be consistently produced in the rat by the formation of blind intestinal segments. 2. Aureomycin appears to be a potent agent in the prevention of this anemia. 3. Ulceration of the blind intestinal segment does not appear to be the major etiological factor in the causation of this anemia.

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2. Barker, W. H. and Hummel, L. E., *Johns Hopkins Hosp. Bull.*, 1939, v64, 215.

4. Butt, H. R., and Watkins, C. H., *Ann. Int. Med.*, 1936, v10, 222.

8. Little, W. O., Zerfas, L. G., and Trusler, H. M., *J.A.M.A.*, 1929, v93, 1290

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Received October 17, 1950. P.S.E.B.M., 1950, v75.

Innate and Acquired Agglutinins in Ducks to the Malaria Parasite *Plasmodium lophurae*. (18336)

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A large proportion of mature female ducks and a small proportion of mature male ducks are relatively resistant to infection by *Plasmodium lophurae*(1,2). It has been shown that the natural or innate resistance of these adult ducks depends at least in part on the presence in their plasma of materials which exert an anti-plasmodial effect when the plasma is injected into young ducklings infected with *Plasmodium lophurae*(2). When preliminary observations revealed that the plasma of uninfected ducks agglutinated free-parasite suspensions of *P. lophurae*(3) and that the degree of agglutination differed with the individual birds, it seemed possible that the agglutinating power of a plasma might be correlated with its protective power when injected into infected ducklings, and with the innate resistance of the duck from which it was obtained. Detailed experiments have not substantiated this possibility, but have indicated a much more complex situation involving the interplay of innate and acquired agglutinins and of unknown factors.

Materials and methods. All the ducks were obtained as day-old ducklings and were fed commercial duck-pellet feeds. They were reared indoors until they were about 3 months old, when they were placed in an outdoor pen until such time as they were to be used in an experiment. The strain (12A) of *P. lophurae* was maintained by the weekly passage of infected blood in ducklings 2 to 3 weeks old. Experimental ducks received intravenously a dosage of blood (taken from a donor on the sixth day of its infection) sufficient to introduce about 25 million parasites per 100 g of body weight. Infections were followed in the usual manner by means of blood films stained with Giemsa's stain. In the experiments with mature or 3- to 4-month-old ducks, a blood sample was taken for agglutinin assay shortly before the ducks were inoculated with the parasites. Samples were taken again on the fourth or fifth day and on the seventh day of the infection. In one experiment with 1-month-old ducklings a portion of the animals was saved by treatment with quinine and additional samples for agglutinin assay were taken over a period which included relapses in a few of the survivors. The details of this experiment will be considered subsequently with the results which were obtained. Ducks were bled from the right jugular vein with heparin solution at the rate of 0.1 ml per 1 ml of blood as an anticoagulant. The heparin solution contained 27 mg of Connaught heparin in 100 ml of 0.85% sodium chloride solution. A 3 ml sample of blood provided enough plasma for a considerable number of agglutination tests, and the sample was limited to this amount whenever frequent tests were to be made on the same duck. When duck plasma was heated for one-half hour at 55° to 56°C a fine precipitate usually formed. In order to use such heated plasma in the agglutination test it was necessary to remove the precipitate by centrifugation. The antigen for the agglutination tests consisted of dense suspensions of *P. lophurae* largely freed from erythrocyte material by the method of Stauber and Walker(3). The suspensions were stored at 4°C. A single preparation was used throughout the course of an experiment. For use, a small amount of material was diluted with enough buffered saline (pH 7.4) to give an opacity corresponding to that of a 1:10 dilution of full-strength material(3).

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[*] Rockefeller Institute, N. Y.

[†] Rutgers University, New Brunswick.

[‡] Visiting Investigator, Rockefeller Institute, 1949-1950.

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2. Trager, W., and McGhee, R. B., *J. Exp. Med.*, 1950, v91, 365.
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The agglutination tests were prepared and read essentially after the manner of Stauber, Richardson and Walker(4). Appropriate dilutions of each plasma to be tested were made in buffered saline. Two drops of plasma or plasma dilution and one drop of dilute antigen suspension were mixed on a clean glass slide by a rotary movement of the slide. The mixture was then incubated at 37°C in a moist chamber for 10 minutes, stirred again and examined under the microscope with a 16 mm objective. Frequent controls were prepared consisting of buffered saline and antigen. These never showed agglutination, but presented a uniform dispersion of fine particles (the parasites) with occasional gross, somewhat flaky, masses which were present in the antigen suspension and could not be confused with a positive agglutination reaction. In the strongest agglutination reaction, which was read as 4+, large clumped masses were present with very little finely dispersed material. Reactions with progressively smaller clumps and more unclumped material were read as 3+ to +. A ± reaction was one which showed very small but still perfectly definite agglutinated clumps in part of the preparation and much unagglutinated material in the rest of it. If mixtures giving any degree of positive reaction after 10 minutes' incubation were allowed to stand several additional minutes at room temperatures, many of them showed a further intensification of the reaction, but mixtures which were negative at ten minutes did not become positive upon longer standing.

Results and discussion. The agglutination reaction. Plasma from ducks which had not been infected with *P. lophurae* always gave the strongest reaction in the preparation with undiluted plasma. The reaction of different plasmas varied from – to 4+. It was usually 2+ to 3+ in ducks 3 months of age or older and + to 2+ in ducklings one month old or younger. The reaction disappeared rapidly with dilution of the plasma. The plasma of most young ducklings showed a negative reaction at 1:2 dilution (1 part of plasma to 1 part of buffered saline). Among the older ducks even plasma which gave a 4+ reaction in the undiluted state rarely gave any positive reaction at a dilution beyond 1:4. A zone phenomenon was not observed. The agglutinating activity of plasmas from uninfected ducks or ducklings was usually completely removed by heating for one-half hour at 55-56°C followed by centrifugation to remove the fine precipitate which formed. The agglutinating activity also tended to decrease with prolonged storage of the plasma in the refrigerator. The addition of guinea pig serum to a heated plasma which had been active in the unheated state did not restore its activity. Plasmas from ducks undergoing an infection with *P. lophurae* varied in their agglutinin titer even more than plasmas from uninfected ducks. With unheated plasma there might be any one of the following patterns: (1) no positive reaction at any dilution, a type of result observed chiefly with very young ducklings; (2) a strong positive reaction with undiluted plasma which rapidly disappeared upon dilution, as with the plasma of uninfected ducks; (3) a negative reaction with undiluted plasma and with plasma diluted 1:2, followed by a positive reaction at higher dilutions, sometimes up to over 1:1000; (4) a positive reaction with undiluted plasma followed by a zone of weak or negative reactions and then by positive reactions up to a high dilution. The 3 latter types of reactions are illustrated in Table I.

Plasmas obtained from infected ducks and subjected to heating for one-half hour at 55-56°C followed by centrifugation usually gave a negative reaction in the undiluted state. If the unheated plasma gave a reaction pattern of type 1 above, then the heated plasma was negative at higher dilutions as well as in the undiluted state. If the unheated plasma gave a pattern of type 2 the heated plasma might be either entirely negative as in uninfected ducks (Table I, duck 776), or it might be positive in the lower dilutions (Table I, duck 973). If the unheated plasma gave a pattern of type 3 or 4 then the heated plasma usually showed a positive agglutination at 1:2 followed by equally strong or even

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4. Stauber, L. A., Richardson, A. P., and Walker, H. A., in press.

TABLE I. Results of Agglutination Tests with Plasma Taken from Ducks on the 7th Day of Infection with *P. lophurae*.

Duck No.	Degree of agglutination at the indicated dilutions of plasma.* (U = Unheated plasma; H = plasma heated ½ hr at 55-56°C and cleared by centrifuging).																	
	Undiluted		1:2		1:4		1:8		1:16		1:32		1:64		1:128		1:256	
	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H	U	H
776	3+	—	2+	—	±	—	—	—	—	—	—	—						
778	—	—	—	±	±	+	±	+	±	+	+	+	+	+	±	+	±	±
780	2+	—	2+	—	±	±	+	±	+	±	±	+						
973	3+	—	2+	±	±	+	—	±	—	—	—	—						
974	+	—	±	—	—	±	+	+	±	+	—	+						
975	3+	—	2+	±	±	+	+	2+	+	2+	+	2+	+	2+	+	+	±	±
987	2+	—	+	±	±	±	—	+	—	+	—	±						
988	3+	—	+	±	+	±	±	2+	±	+	+	+						

* Blank space indicates no test made.

stronger reactions at higher dilutions and going out to as great a dilution as that of the unheated plasma (Table I, ducks 778, 975). Sometimes the reaction of heated plasma at the higher dilutions was stronger than that of the same plasma unheated. Heat stable agglutinins were not observed in the plasmas of a series of ducklings inoculated when they were one month old. The positive agglutinations seen with heated plasma differed in character from those with unheated plasma. Heated plasmas have never at any dilution tested given more than a 2+ reaction, and usually they gave a + or ± reaction. This was true even of plasmas which still gave a ± reaction at a dilution of 1:1024. Positive unheated plasmas differed from each other chiefly in the degree of agglutination shown by the undiluted plasma, which ranged from the formation of very small clumps to the formation of very large ones easily seen macroscopically. Positive heated plasmas on the other hand generally formed only small clumps throughout their positive range, and differed from each other chiefly in the extent to which they could be diluted and still show agglutination.

These results may be interpreted in terms of innate heat-labile agglutinins and acquired heat-stable agglutinins. The latter are probably of the nature of the usual antibodies which develop in response to antigenic stimuli. Ducks are generally poor antibody producers (5). Nevertheless the plasma of some mature and 3- to 4-month-old ducks which had been infected with *P. lophurae* showed heat-stable agglutinins for free parasite preparations of this species even at a dilution of 1:1024. Agglutinating antibodies to such preparations of *P. lophurae* have been produced in rabbits by injecting them with infected duck blood(4). The innate agglutinins are possibly similar to the heat-labile components of normal serum which have been observed to react against other infectious agents such as Toxoplasma (6) and some of the viruses(7).

The changes in the heat-labile and heat-stable agglutinins during the course of infection in mature and 3- to 4-month-old ducks. The results of a typical experiment are presented in Table II. In this experiment and 2 others like it totals of 8 mature females, 8 mature males and 8 3- to 4-month-old ducks were used. Of these, 7 females, 1 male and 3 immature ducks developed infections with a peak parasitemia of less than 100 per 1000 red cells, confirming again the relatively greater resistance of the mature females(1,2). There was no correlation between the strength of the agglutination reaction exhibited by the plasma before inoculation and the subsequent course of the infection. Except in some very light infections (Table II, duck 990) the reaction given by the unheated, undiluted plasma decreased as the infection progressed. Ducks which showed this change to a marked

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6. Sabin, A. B., and Feldman, H. A., *Science*, 1948, v108, 660.

7. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, v90, 475.

TABLE II. Course of the Parasitemia and Changes in the Heat-Labile (U) and Heat-Stable (H) Agglutinins in Ducks Infected with *P. lophurae*.

Duck			Parasites per 1000 red cells on days after inoculation						Degree of agglutination (— to 4+) given by undiluted, unheated plasma (U), and highest dilution of heated plasma giving a positive agglutination (H) on days after inoculation					
									0		5		7	
No.	Sex	Age, mo.	4	5	6	7	8	9	(U)	(H)	(U)	(H)	(U)	(H)
744	♀	7	30	11	0.1	0	0	0	+	0	2+	1:64*	+	1:64*
747	♂	7	122	468	784	1592	D		3+	0	+	1:4	—	1:2
751	♀	7	26	66	368	546	1125	1340	2+	0	+	1:8	—	1:4
781	♀	3	160	380	212	484	434	252	3+	0	+	1:64*	—	1:64*
784	♂	3	44	74	66	2	4	5	2+	0	3+	1:64*	+	1:64*
785	♀	3	116	586	780	1535	D		3+	0	2+	0	+	1:4
957	♂	13	76	201	112	344	310	406	3+	0	—	1:64*	±	1:64*
990	♀	12	4	2	0.2	0	0	0	3+	1:4	3+	1:32	3+	1:64*

D denotes death. * Highest dilution tested.

degree and which, in addition, developed only a low titer of heat-stable agglutinins, also showed the most severe infections (Table II, ducks 747, 751, 785). A few of the resistant ducks maintained a high level of heat-labile agglutinins and developed no heat-stable agglutinins, but most of them also acquired the heat-stable agglutinins. In moderately susceptible ducks, the heat-labile agglutinins decreased and heat-stable agglutinins appeared in relatively high titer (Table II, ducks 781, 957). The decrease in heat-labile agglutinins which occurs in the more susceptible older ducks and in all young ducklings could as well be a result rather than a cause of the concomitant high parasitemia. It is noteworthy that complement similarly undergoes a marked decrease in titer during the course of infection of chicks and ducklings with *P. lophurae*(8,9).

Changes in the heat-labile agglutinins of ducklings during infection with P. lophurae *and during latency and relapse.* A number of observations with young ducklings indicated that their heat-labile agglutinins disappeared completely at the peak of infection and that they did not develop heat-stable agglutinins. The following more detailed experiment confirmed these observations and provided some additional information concerning a possible relation of the agglutinins to relapse. A uniform batch of 24 ducklings was used. When these were 25 days old a blood sample was taken from each for determination of the agglutinins. Two days later 19 of the ducklings were inoculated with *P. lophurae*. Blood films from these ducklings were prepared daily from the second to the 8th day after inoculation, and at 3- to 4-day or sometimes shorter intervals thereafter. Twelve of the inoculated ducklings were treated with quinine on the 5th, 6th and 7th days of their infection. Each treated duckling received by stomach tube enough of a 5% solution of quinine dihydrochloride to supply it with 10 mg of the drug per 100 g of body weight. Blood samples for agglutinin determinations were taken from all the ducklings (including the 5 uninfected ones) on days 2, 4 and 7 after the day of inoculation, and from the survivors on days 19, 28, 40, 49 and 56.

Although all the plasmas were tested at several dilutions for heat-stable agglutinins, none were observed. Positive reactions with unheated plasma were rare at dilutions beyond 1:2. Hence it will be sufficient to consider only the degree of agglutination with the unheated, undiluted plasma. Among the 5 uninfected ducks, 2 gave a + and 3 a 2+ reaction in the first sample taken at 25 days of age. Most of the later samples were + or 2+. In the last samples taken when the ducklings were 83 days old, 3 showed a 2+ and 2 a 3+ reaction. Among the 7 ducks which were infected and not treated with

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8. Trager, W., *J. Exp. Med.*, 1947, v85, 663.
9. McGhee, R. B., unpublished results.

TABLE III. Changes in Heat-Labile Agglutinins of Ducklings During Infection with *P. lophurae* and During Latency and Relapse. Ducklings inoculated when 27 days old. On the 5th to 7th days of infection each duckling received by stomach tube quinine dihydrochloride (as a 5% sol.) 10 mg per 100 g of body wt. See text for results with uninfected control ducklings and with infected ducklings not treated with quinine.

	Parasites per 1000 red cells on days after inoculation															Degree of agglutination by undiluted plasma on days after inoculation								
No.	6	7	8	11	14	19	25	28	32	37	40	42	46	49	56	−2	2	4	7	19	28	40	49	56
129	240	260	225	3	0.5	0	0	0.2	0.6	45	436	1180D				3+	3+	3+	+	4+	2+	±		
130	498	370	332	27	0	0.5	0.1	0.1	0.8	0	0	0	0	0	0	±	2+	2+	−	3+	3+	3+	3+	3+
135	254	196	168	29	7	1220D										+	3+	3+	+	−				
136	440	478	486	12	0	0	0	0	0	0.1	0.1	0	0	0	0.1	+	3+	2+	±	3+	2+	3+	3+	3+
137	404	388	186	0	0.5	4	0	0	0	69	1055	D				±	2+	+	+	−	−	−		
138	220	124	5	0	0	0.1	0	0	0.2	0.3	1	2	12	0.7	0.1	±	2+	3+	2+	+	+	±	2+	2+

D denotes death on or just after indicated day. See text.

quinine, four had a +, two a 2+ and one a 3+ reaction in the initial samples taken 2 days before inoculation. Two days after inoculation all 7 had a 2+ reaction and this was essentially the case 4 days after inoculation, but on the 7th day all were entirely negative. The ducks died on the 8th day. Among the 12 infected ducks treated with quinine 4 died on the 9th day and 2 others a few days later, in spite of some reduction in parasitemia effected by the quinine. The course of events in the 6 ducks which survived to be sampled on the 19th day after inoculation is shown in Table III. All of these ducks showed a drop in the agglutination reaction on the 7th day. In 2 (ducks 130, 136) this drop was followed by an increase which persisted in subsequent samples, and few or no parasites were found in the blood films. A 3rd duck (129) exhibited an increased reaction on the 19th day followed by a decrease to ± on the 40th day. The decrease was accompanied by a severe relapse which ended in death on the 43rd day. In duck 138, a slowly decreasing reaction was accompanied by a slowly increasing but low parasitemia which reached a peak around the 46th day and then fell off, while the agglutinin reaction rose to 2+. Duck 135 with a negative reaction on the 19th day was then at the peak of a relapse and died on the following day. The 6th survivor (137) had a negative reaction on the 19th day and also in the 2 subsequent samples. It developed a severe relapse and died on the 42nd day. The 28-day sample of blood from this bird contained more than half as great a volume of white blood cells as of red cells. These white cells appeared to be hemocytoblasts and monocytes. They were present in smaller numbers at 32 days, just before the malarial relapse began. Subsequent samples, taken during the relapse, presented only the usual blood picture.

Summary. Erythrocyte-free suspensions of *Plasmodium lophurae* were agglutinated to a variable degree by the fresh plasma of ducks which had not been infected by this parasite. The agglutinating activity of the plasma was in general higher in older ducks than in young

ducklings. It was rarely evident beyond a 1:4 dilution of the plasma, and was fully removed by heating the plasma for one-half hour at 55-56°C followed by centrifugation to remove the precipitate which formed. In infected ducks 3 months of age or older these heat-labile agglutinins generally decreased as the infection progressed, except in individuals which exhibited a strong innate resistance to the parasites. The heat-labile agglutinins disappeared completely at the peak of a severe infection. Heat-stable agglutinins appeared in the plasma of most of the infected birds. These, unlike the heat-labile agglutinins, were not demonstrable in undiluted plasma but often were active in plasma diluted as far as 1:1000. In ducklings infected when they were one month old or younger, heat-stable agglutinins did not appear. As in severely infected older ducks, the heat-labile agglutinins vanished at the peak of the infection. If such ducklings were saved from death by treatment with quinine the heat-labile agglutinins reappeared to a varying degree. Relapses, like initial infections, were accompanied by a lack of heat-labile agglutinins.

Received October 18, 1950. P.S.E.B.M., 1950, v75.

Experimental Studies on Pathogenesis of Hemoglobinuric Nephrosis.

(18337)

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The exact mechanism of the production of the renal lesion following the transfusion of incompatible blood is as yet unclear. It has become apparent that no one factor is responsible, but probably some combination of hemoglobin excretion, the pH of the urine, shock (renal anoxia), and the incompatible reaction *per se* is involved. Many factors have been investigated in the production of hemoglobinuric nephrosis. Among these factors are the "toxicity" of hemoglobin(1-11), the chemical form of the excreted hemglobin (7,12-14), the pH of the urine(1,6,13,14), dehydration(8,9,11,14,15), renal anoxia(16, 17), hemorrhagic shock(2,5), subcortical renal vascular shunt(18), and the incompatible transfusion reaction *per se*(19-21). It has been suggested(22,23,25) that hemo-

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TABLE I. Summary of Experiments, Giving Amount of Blood Removed, Blood Pressure Level During Shock, and Amount of Hemoglobin Infused.

Hemolysed blood	Dog No.	Total amt blood removed (cc/kg)	Approx. avg blood pressure level during shock (mm Hg systolic)	Amt hemoglobin infused (g/kg)	Time elapsed from hemoglobin infusion to full recovery (hr)
Without red blood cell stroma	1	44	60	0.9	20
	5	40	50	1.0	(died 40 min.)
	4	48	53	0.5	20
	6	44	55	0.6	20
	2	49	45	0.6	12
	1	40	48	0.6	8
With red blood cell stroma	7	50	48	1.0	(died 9 hr)
	8	40	53	0.9	12
	4	43	50	0.5	18
	8	40	50	0.7	(died 4 hr)
	12	34	54	0.6	10
	9	47	50	0.7	10
	14	46	54	0.7	(died 17 hr)
Avg		43.4	52	0.7	

globinuric nephrosis may be due to a combination of shock and hemoglobinuria. Two other factors of importance are dehydration and an acid urine, and in hemorrhagic shock both are present(5). Hence it seemed logical to use a procedure that combines all these known factors (shock, hemoglobinuria, acid urine and dehydration). This experimental procedure, moreover, reproduces in most part the sequence of events as it occurs in many human cases of hemoglobinuric nephrosis. Therefore, it was decided to produce hemorrhagic shock of sufficient duration and depth to produce renal ischemia, to follow this by an infusion of hemoglobin solution, and then to bring the animals out of shock by transfusion with their own blood.

Method. Two groups of dogs were used, one receiving dog blood, the red blood cells of which were lysed and the red blood cell stroma removed, and the other receiving hemolysed dog blood containing the red blood cell stroma. Three dogs were used twice. Dog No. 1 was used 2 times in the group receiving hemolysed blood with stroma, dog No. 8 2 times in the group receiving hemolysed blood without stroma, and dog No. 4 once in each group. At least 5 weeks elapsed before the procedure was repeated on any dog. An average of 0.7 g/k of hemoglobin (range of 0.5-1.0) was given; this approximates the amount liberated in one incompatible transfusion in the human. (About 1.0 g/k in a 70 kg man.) Hemoglobin solutions were obtained by rapid freezing of citrated dog blood at –20°F, and thawing just before use. Female mongrels were anesthetized with pentobarbital sodium, and put in hemorrhagic shock. At the end of 40 minutes(24) the prepared hemoglobin solution was infused; this was followed by transfusion of their previously removed citrated blood except for an amount equal to the volume of hemoglobin solution infused (Table I). Blood pressures were recorded throughout most of the transfusion period. Urinalyses and non-protein-nitrogen determinations were performed pre- and post-operatively.

Results. Thirteen dogs were used, 6 in the group receiving hemolysed blood without red cell stroma, and 7 in the group receiving hemolysed blood with stroma. One of the former group and 3 of the latter failed to survive. Of those animals that survived, the results in each group were similar. The in-

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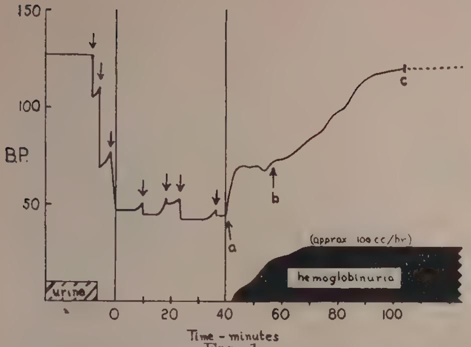

FIG. 1.

This diagram represents a typical recording of blood pressure (systolic, mm Hg) and urine flow during the procedure described in the text. Downward arrows indicate bleeding, the first such arrow representing the largest blood loss. The hemorrhagic shock period extends from 0-40 min. 'a'—start of hemoglobin solution infusion; 'b'—conclusion of hemoglobin infusion and start of citrated blood; 'c'—kymographic tracing discontinued.

fusion of the hemolysed blood was followed by an immediate rise in blood pressure to an average of 80 mm Hg systolic where it remained for about 10 minutes. A second slower rise occurred after a small amount of normal blood was infused, following which the blood pressure returned to normal in 15-20 minutes (Fig. 1). Return to full reactivity occurred within a period of 3-5 hours, and all animals were completely well clinically within 20 hours.

Flow of urine ceased at the onset of the shock period, and resumed in the post-shock period as soon as the blood pressure rose to 75-80 mm Hg systolic. At this time the urine was dark red-brown and remained so for 4-6 hours. For 5-6 hours after the infusion of the hemoglobin solution there was a marked diuresis, yielding as much as 100 cc/hr. All pre- and post-operative urines were acid in reaction. The latter were usually dilute (1.004-1.010) but occasionally animals excreted urines of specific gravity of 1.028-1.034. The exact amount of hemoglobin excreted was not determined. All post-operative urines contained 4+ protein; none contained glucose. Histologic examination of the sediment revealed granular, brown, amorphous material, and rare granular casts. The urine was completely normal within 24 hours. The blood non-protein-nitrogen ranged from 31-48 mg/100 cc pre-operatively, and from 38-48 mg/100 cc post-operatively; in no instance was there any significant elevation within one week after the procedure.

All the animals that died did so within 17 hours of the procedure. The death in the group receiving hemolysed blood without red blood cell stroma was probably due to anesthetic complications. Three dogs died in the group receiving hemolysed blood and red blood cell stroma. It is probable that these animals died in so-called 'irreversible' shock according to Wiggers' criteria(27). Autopsies on these 4 dogs revealed marked congestion of all the viscera and vascular engorgement. Histologic examination of the tissues was unremarkable except for congestion. The kidneys contained numerous hemoglobin casts in the tubules without evidence of tubular degeneration.

Three of the surviving dogs were sacrificed 2, 4, and 5 weeks respectively after the procedure and none revealed any gross or histologic evidence of any renal lesion.

Discussion. Thirteen dogs received hemolysed blood with or without the red blood cell stroma following a period of hemorrhagic shock. The length and depth of hemorrhagic shock employed was probably sufficient to produce almost complete cessation of renal blood flow and resultant renal stagnant anoxia (17). The fact that 4 dogs died of 'irreversible' shock is additional evidence that the hemorrhagic shock was of maximal length and depth compatible with survival. Despite this, none of the 9 surviving dogs developed any evidence of hemoglobinuric nephrosis, and the 4 dogs that died revealed no morphologic evidence of a renal lesion. The reasons for the failure to produce hemoglobinuric nephrosis could be inherent in the technic. Larger doses of hemoglobin, severe dehydration, and marked acidosis could be added alone or in combination to the described method. It is our belief, however, that those who have

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produced renal lesions comparable to hemoglobinuric nephrosis by using these extreme measures have gone beyond the rational pathogenetic sequence of events which usually occurs in the production of hemoglobinuric nephrosis in humans.

An important factor that is present in the production of this lesion in humans but was not included in our experiments is the incompatible transfusion reaction. True transfusion reactions in dogs, however, have been produced(22). None of these animals developed any evidence of hemoglobinuric nephrosis. It is possible, then, that some factor in the incompatibility reaction, plus hemoglobinuria, in addition to prolonged renal anoxia and/or hemorrhagic shock may be responsible for the production of such a lesion. At present we are investigating this line of reasoning.

Summary and conclusions. Prolonged hemorrhagic shock was produced in 13 dogs. This was followed by infusions of hemolysed blood with or without red blood cell stroma. The animals were then brought out of shock with transfusions of their own blood. Four dogs died within 17 hours of anesthetic complications or 'irreversible' shock. The 9 surviving dogs revealed no evidence of hemoglobinuric nephrosis, and were completely well within 20 hours. In this study the combination of prolonged hemorrhagic shock and hemoglobinuria failed to produce hemoglobinuric nephrosis.

Received October 23, 1950. P.S.E.B.M., 1950, v75.

Nucleoprotein Content During the Staphylococcal Growth Cycle.* (18338)

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The key role played by nucleic acids in the processes of normal growth and neoplasia has been well demonstrated in recent years by the work of Caspersson and his group(1). Bacterial cultures offer excellent systems in which to trace the changes in nucleic acid concentrations with growth and it is the object of the present study to determine the concentrations of desoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) at significant stages in the growth cycle of *Staphylococcus aureus*. Previous investigations of the nucleic acid contents of staphylococcus have been sparse and somewhat contradictory. Thus, Vendrely and Lehoult(2) determined the nucleic acid contents of *Staphylococcus aureus* at a single growth stage (*i.e.* 20 hour cultures on agar) and found the PNA content to be 3 times as high as the DNA content. Price(3) in an analysis of a 22 hour slant of *Staphylococcus muscae* reported equal amounts of PNA and DNA, although in earlier phases of the growth cycle the PNA concentration was 2-2½ times that of DNA.

Methods. Determinations were carried out on *Staphylococcus aureus* of the "L" strain from the Department of Microbiology of Boston University School of Medicine. All cultures were grown in a medium prepared by adding water to 15 g of Bacto-Casamino Acid and 5 g of Bacto-Yeast Extract to a total volume of 1 liter. The medium was adjusted to pH 8 and autoclaved at 10 lb pressure for 20 minutes. In order to have reproducible lag period behavior, it was necessary to have an inoculum in a resting or decay stage but which contained a minimum of nonviable bacteria, since in the lag phase samplings up to one hour contain the same per cent of nonviable bacteria as the inoculum. Similarly, the inoculum must be large enough to come within range of the chemical technics employed. It was discovered that an inoculum large enough to allow accurate duplicate determination within the first hour was too large to permit normal growth in the logarithmic phase. Inocula were prepared by incubating for 24 hours 1 ml of a previous culture in 100 ml of medium in 250-ml Erlenmeyer flasks at 37°C. For lag phase determinations, 50 ml was spun down in a refrigerated centrifuge and inoculated into 500 ml of medium. For logarithmic phase determinations, 25 ml was so treated. The growth curve was followed by means of turbidity measurement at a wave length of 600 mμ on a Coleman Junior Spectrophotometer. Samples of 100 to 200 ml were removed at selected times, spun down, washed with distilled water and made up to 20 ml. Aliquots were removed for total nitrogen determinations. The samples were then washed with cold 10% trichloracetic acid (which, by inactivating nuclease activity, preserves the nucleic acid molecule relatively intact) and with 95% alcohol. It was found desirable to modify the washing procedure by resuspending the centrifuged bacteria in 1 or 2 ml water before adding the trichloracetic acid or alcohol to prevent clumping. The bacteria were stirred for 10 minutes with each washing. The samples were then hydrolyzed with two 5-ml portions of 5% trichloracetic acid in a water bath at 90°C. The hydrolysates were combined and centrifuged a second time to remove particulate matter and made up to 18 ml with 5% trichloracetic acid. Aliquots were now taken for determination of PNA, DNA, nucleic acid P, and nucleic acid N.

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* The research reported in this paper was made possible through support extended to Boston University by the Navy Department (Office of Naval Research under contract No. N6ori-160,) and by the United States Public Health Service.

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PNA was measured by the orcinol method (4), photometric measurement being made at a wave length of 670 mμ. The procedure was tested by measuring known mixtures of PNA and DNA standards, a correction factor(5) being calculated on the basis of an orcinol-DNA curve. DNA was measured by the diphenylamine method(6), the photometric readings being made at 600 mμ. Commercially prepared PNA and DNA of Nutritional Biochemical Laboratories were used as standards in these two reactions. The preparations were about 90% nucleic acid as calculated from phosphorus determinations. The impurity was determined chromatographically to be largely or entirely adenine, which does not interfere with either the orcinol or the diphenylamine tests. No free phosphate was found. Phosphorus was determined according to the method of Fiske and Subbarow(7). Total nitrogen and nucleic acid nitrogen were measured as ammonia by Nesslerization. The reagent used in Kjeldahl digestion was 1 ml of selenium oxide and 1 ml of a saturated solution of cupric sulfate made up to 100 ml with concentrated sulfuric acid. The photometric measurements were carried out at a wave length of 520 mμ.

Results. The significant data obtained are shown in Table I. The nucleic acid values (in Columns III and IV) have been related to total nitrogen of whole bacteria washed with distilled water. The ratio of nucleic acid N to nucleic acid P (Column V) tends to be somewhat higher than the theoretical 1.7. Total nucleic acid content of the inocula are determined in two ways: by calculation from the nucleic acid P (Column VI); and by addition of the PNA and DNA content as determined colorimetrically (Column VII). Price(3) reports agreement between nucleic acid colorimetry and phosphorus determinations within ± 15%. Ten out of the thirteen sets of determinations agree within this range.

Discussion. Our values agree with those of Price(3) on *Staphylococcus muscae* in having equal amounts of the 2 nucleic acids in a

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TABLE I. Pentosenucleic Acid and Desoxypentosenucleic Acid Values for *Staphylococcus aureus* of the "L" Strain Over the Early Stages of the Growth Cycle.

Sampling time (min.)	Incr. of optical density at time of sampling	DNA/ total N	PNA/ total N	Nucleic acid N / Nucleic acid P ratio	Total nucleic acid (mg)	
					From P values	PNA + DNA
0	—	.64	.60	1.68	.22	.15
0	—	.59	.49	1.61	.28	.15
0	—	.59	.72	2.50	.16	.14
37	.016	.51	1.02	2.05	.21	.17
40	.020	.62	1.47	1.83	.18	.20
55	.024	.69	1.04	2.45	.14	.14
55	.039	.67	.95	1.80	.21	.21
70	.024	.86	1.44	1.61	.19	.20
90	.056	.76	1.54	1.95	.22	.25
93	.036	.81	1.54	2.10	.19	.20
125	.089	.65	1.41	1.81	.22	.21
185	.089	.80	1.34	2.00	.20	.21
185	.091	.67	1.15	2.00	.22	.23

resting culture. It will be noted, however, that as growth proceeds, both the DNA and PNA concentrations increase, the latter more sharply, to a peak at 1-1½ hours of growth, after which declines take place. The values of Vendrely and Lehoult(2), related to mg of total nitrogen, are 0.625 mg PNA and 0.2 mg DNA. Their very low DNA value suggests that one extraction fails to remove all the relatively insoluble DNA.

Summary. The concentrations of pentosenucleic acid and desoxypentosenucleic acid per total nitrogen content at significant stages in the growth cycle of *Staphylococcus aureus* were determined. The concentrations were equal in resting or decay cultures, rose to a peak at 1-1½ hours of growth and thereafter declined. At peak concentrations, the PNA content was about twice that of DNA.

Received October 26, 1950. P.S.E.B.M., 1950, v75.

Effect of Plaster Cast on Tourniquet Shock.* (18339)

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Experimental tourniquet shock in rats is probably initiated by local fluid loss(1). The following study was made to determine whether shock could be delayed or prevented by applying a cast to reduce the edema. Previous reports indicate that local pressure is beneficial for both rats and dogs in tourniquet(2-4) and traumatic shock(5).

Method. This has been described previously (1). The technic results in severe shock which causes death within a few to several hours. Adult white male rats weighing about 200 to 250 g were used. Circulation in the left hind extremity was occluded for 5 hours by tightly wound rubber bands. Food and

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TABLE I. Survival Time (Hr).

	No.	Mean	Stand. dev.
Cast rats	25	10.5	±3.6
Control rats	19	4.5	±6.9

water were withheld for about 8 to 10 hours prior to application of tourniquet. Immediately after the tourniquet was removed, a plaster cast was placed around the extremity and carried well up on the abdomen. The cast was wrapped over gauze without exerting pressure. If applied too tightly, there is interference with circulation and the limb subsequently becomes necrotic and infected. In shock experiments where local pressure is used to prevent edema, the objectives are to preserve both the extremity and the animal. Cast rats and control rats without cast were compared as to mortality, survival time, fluid loss, hemoconcentration and serum creatine. Fluid loss was obtained by the method of bisection and the results were expressed as percentage of total body weight. Hemoconcentration was measured by hematocrit. Serum creatine was determined by a modification of the method previously described(1).

Results. Survival times are given in Table I. Cast rats lived longer than the controls, *i.e.* a mean of 10½ as compared to 4½ hours. The mean standard error of the two groups was 1.8 and the ratio of the mean difference to the mean standard error was 3.3.† Statistically, this is highly significant. However the mortality in both groups was 100%.

Table II shows fluid loss in cast and control animals at intervals up to 5 hours. The mean standard errors of the two groups were 0.35 and 0.31 at 1 hour and 3 hours respectively and the ratios of the mean difference to the mean standard error were 3.1 and 5.5 respectively. These results are of high order of statistical significance.

The effect of the cast was to diminish the rapidity of local extravasation. Cast rats required a period of 4 to 5 hours to lose as much fluid as did rats without cast in about one hour. However, at the time of death fluid loss in 21 cast rats ranged from 3.2 to 5.9% of body weight and the mean was about 4.3%. These figures are almost of the same magnitude as in non-cast rats dead in a shorter time and appear sufficient to explain the origin of shock. This in no way excludes the presence of a toxic factor. Thrombosis of vessels in the casted extremity was not observed at autopsy. Hemoconcentration occurred more rapidly in the control than in the cast rats as shown in Table III.

The rise in serum creatine was distinctly less pronounced in the cast rats (Table IV). At 3 and 5 hours respectively after removal of the tourniquet, the values in non-cast rats were 50 and 100% higher than those in the cast animals. The mean standard errors of the 2 groups at 1, 3, and 5 hours respectively were 1.7, 2.4, and 2.2. The corresponding ratios of the mean difference to the mean standard error were 4.7, 6.1, and 15.1. These are statistically significant results.

Discussion. In this study a rigid cast, applied to the extremity of a rat immediately after prolonged ischemia, did not prevent edema. The cast limited swelling of the limb and altered the direction of extravasation. Fluid was shifted upward into the fascial planes between subcutaneous tissue and oblique abdominal muscles. When the cast was removed after death, no significant swelling of the extremity was visible grossly, although there was marked internal edema of the lower abdominal wall and flank. The latter sites cannot be restricted by the cast. Such fluid loss is readily detected by the method of bisection, whereas immersion (fluid displacement) would not be accurate.

Another effect of the cast was to diminish the rapidity of local fluid loss. This may have been due in part to the resistance offered to the extravasate by the soft tissues of

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† The stand. dev. was calculated by the formula $\sigma = \frac{\Sigma\chi^2}{n-1}$ where $\Sigma\chi^2$ represents the sum of the squares of the deviations from the mean and n is the number of animals. The mean stand. error was determined by the formula $\sqrt{\frac{(\sigma_1)^2}{n_1} + \frac{(\sigma_2)^2}{n_2}}$, where σ_1 and σ_2 are the stand. dev. of the cast and control rats respectively, and n_1 and n_2 the number of animals in each group. When the ratio of the mean difference to the mean stand. error is greater than 2.5, the result is significant at the 1% level.

TABLE II. Fluid Loss.

Time of sacrifice after release of tourniquet,* hr	% of body weight			
	Cast rats		Control rats	
	Mean	Stand. dev.	Mean	Stand. dev.
1	2.3	±.79	3.4	±.32
3	3.1	±.93	4.8	±.58
5	3.5	±.76		

* 12 cast and 15 control rats were sacrificed at each time interval.

TABLE III. Hemoconcentration.

Time of sacrifice after release of tourniquet,* hr	Mean incr. in hematocrit	
	Cast rats	Control rats
1	12	14
3	14	23
5	18	

* Determinations were made on 15 cast and 15 control rats at each time interval.

TABLE IV. Serum Creatine.

Time of sacrifice after release of tourniquet,* hr	Serum creatine			
	Cast rats		Control rats	
	Mean	Stand. dev.	Mean	Stand. dev.
1	22.1	±3.6	30.1	±5.0
3	26.2	±7.1	40.9	±3.4
5	30.2	±5.5	63.5	±3.9

* 11 cast and from 15 to 25 control rats were sacrificed at each interval. In the 3 and 5 hour controls, determinations were usually made on pooled blood from 2 or 3 animals.

the abdominal wall after further expansion of the leg was prevented. The slower rate is consistent with prolongation of survival time, even though mortality is not altered. At the time of death, fluid loss in cast rats was comparable to that in the non-casted controls. Cast rats also showed a slower rate of tissue breakdown, as measured by serum creatine, than control animals. Possibly this, as well as slower fluid loss and hemoconcentration, was related to altered circulation in the extremity after a cast was applied, *i.e.*, blood might pass through the main capillary bed without use of bypaths. Also, since elevation of serum creatine results from washing out of muscle creatine after anoxia causes irreversible hydrolysis of phosphocreatine, the slower rise might indicate reduced blood flow.

In this study edema developed in spite of the cast. The impression was gained that after prolonged anoxemia of a limb it is not possible to apply a rigid cast which prevents edema while still permitting normal blood flow. On the other hand Glenn, Gilbert and Drinker(7) claimed that a cast applied to the foot of dogs after a severe burn allowed normal circulation and abolished capillary leakage.

Summary. Tourniquet shock in rats cannot be prevented by a rigid cast applied to the hind extremity immediately after removal of the tourniquet. The effect of the cast is to reduce the rapidity rather than the ultimate magnitude of local fluid loss. Survival time of the animals is prolonged but there is no decrease in mortality. The development of shock is accompanied by hemoconcentration and rise in serum creatine both of which occur more gradually than in control rats without casts.

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Received October 23, 1950. P.S.E.B.M., 1950, v75.

Lithospermum ruderale and the Incidence of Mammary Tumors in Mice.* (18340)

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Mammary tumors in mice are thought to develop "spontaneously" as the result of 3 concomitant factors: (a) an inherited susceptibility, (b) the milk agent and (c) hormonal stimulation(1). All 3 components are necessary for the development of such tumors. Therefore, any procedure which would interfere with the action of one of these factors should interfere with the development of mammary tumors by breaking the chain of events. *Lithospermum ruderale* is a plant which induces diestrous by inhibiting the action and/or formation of gonadotropic hormones(3-8). Thus supposedly, the formation of estrogenic hormones is inhibited. For this reason, a study of the effect of lithosperm on the incidence of mammary tumor formation in susceptible mice was undertaken.

Exp. I. C_3H female mice, which as virgins have a high incidence of mammary tumors, namely 63.6% at 402 days(2), were used. Twenty-two virgin females, about 3 months of age, were caged individually. Estrous smears were taken daily, except Sundays, by the lavage method. After a control period of 30 days, during which time regular estrous cycles were recorded in all mice, they were fed various concentrations of ground lithosperm mixed with ground Purina fox chow to determine the concentration of lithosperm necessary to cause diestrous. Six of 11 mice given 15% lithosperm for 35 days went into diestrous, five having occasional estrous cycles. Increasing the concentration of drug to 20% for 40 days had little added effect on estrous cycles. These mice were then used for other purposes. Another 11 mice received 30% lithosperm diet for 85 days. No estrous cycles occurred. Reducing the concentration to 20% for 30 days caused 3 mice to develop one or 2 estrous periods. They were then returned to a 30% lithosperm diet for the rest of their lives. None of the 11 went into estrous again and none developed mammary tumors. These mice died 3 to 14 months (average 11 months) after lithosperm diet was started, or at an average age of about 15 months. The antiestrous effect of lithosperm was not due merely to added bulk in the diet for neither 30% powdered cellulose (alphacel) nor 30% lithosperm marc (after extraction to remove the active principle) induced diestrous in C_3H mice.

Exp. II. Sixty-seven C_3H virgin female mice, 6 to 8 weeks of age, were individually caged and divided into 2 groups: 31 control mice, receiving ground Purina fox chow *ad libitum*, and 36 treated mice, receiving drug diet *ad libitum* which consisted of ground Purina fox chow plus ground lithosperm. The concentration of lithosperm was 15% for 4 weeks, 20% for 8 weeks and then 30% for the duration of the experiment. Mice were weighed once a week, at which time they were examined carefully for the presence of

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* This work was supported by grants from the U. S. Public Health Service and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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TABLE I. Incidence of Mammary Tumors in C_3H Mice.

Group	No. mice	No. dev. tumors	Avg tumor age in days	No. dying without tumors	Avg non-tumor death age in days	No. survivals
Control	31	18	413	12	483	1
Treated	36	1	378	32	483	3

TABLE II. Body Weights of C_3H Mice.

Age in days	Avg body wt in g Control	Treated	% diff.
49	14.5	14.9	+ 2.7
119	21.1	19.8	— 6.1
189	23.6	19.9	—15.7
259	24.3	19.4	—20.1
329	25.2	19.4	—23.0
399	24.6	18.3	—25.6
469	25.9	17.6	—32.0

mammary tumors. The experiment was terminated after 90 weeks. Surviving mice were then 686 days old.

Results are recorded in Tables I and II. In the control group, 18 of 31 mice (58.1%) developed mammary tumors at 287 to 616 days of age, average tumor age being 413 days. Twelve mice (38.7%) died without tumors at an average age of 483 days. One mouse was alive and tumor free (grossly) at the end of the experiment. In the treated group, only one of 36 mice (2.8%) developed a mammary tumor at 378 days of age, thirty-one (88.8%) died without tumors at an average age of 483 days and three (8.3%) were alive and tumor free at the end of the experiment.

Daily food intakes of 18 treated and 14 control mice were recorded for a period of 10 days when the mice were 390 days old. At this time control mice, weighing an average of 26.0 g, ate an average of 4.51 g food per mouse per day, or 17.34 g per 100 g body weight per day. Treated mice, weighing an average of 18.85 g, ate 4.25 g diet, of which 30% was lithosperm giving a fox chow intake of 2.98 g per mouse per day, or 15.8 g per 100 g body weight per day. Thus this group of treated mice weighed 28% less than controls and ate 34% less fox chow on the basis of intake per mouse per day, or 9% less on the basis of intake per 100 g body weight. These figures disregard whatever food value was obtained from lithosperm.

Exp. III. Twenty first generation hybrids (ZD_8F_1) of C_3H females mated with D_8 males were studied for the effect of lithosperm on estrous cycles. ZD_8F_1 virgin females have a high incidence of mammary tumors, namely 96.4% at 355 days(2). Twenty percent, 30% and 40% lithosperm drug diet did not alter the frequency of estrous in this strain of mice. Also subcutaneous injection of an extract of lithosperm which induced diestrous in C_3H mice had no effect in ZD_8F_1 mice in up to 4 times the dose effective in C_3H mice. Therefore ZD_8F_1 mice are less sensitive to the anti-estrous effect of lithosperm than C_3H mice.

Exp. IV. ZD_8F_1 virgin females, at 6 weeks of age, were divided into 2 groups: 30 were given control diet, ground Purina fox chow, *ad libitum*; 32 were fed *ad libitum* on ground Purina fox chow mixed with lithosperm, 15% for 4 weeks, 20% for 8 weeks and then 30% for the remainder of the experiment (448 to 497 days). Usually 5 mice were housed together in a cage. Body weights were recorded once a week, at which time the mice were examined carefully for the presence of mammary tumors. Results are recorded in Tables III and IV. Twenty-nine of 30 control mice (96.6%) developed tumors at an average age of 371 days (217 to 504). Seven of 32 treated mice (21.9%) developed tumors at an average age of 406 days (224 to 518). Again

TABLE III. Incidence of Mammary Tumors in ZD_8F_1 Mice.

Group	No. mice	No. dev. tumors	Avg tumor age in days	No. dying without tumors	Avg non-tumor death age in days	No. survivals
Control	30	29	371	0	—	1
Treated	32	7	406	3	413	22

TABLE IV. Body Weights of ZD_8F_1 Mice.

Age in days	Avg body wt in g Control	 Treated	% diff.
42	15.8	16.3	+ 3.1
112	22.2	21.1	— 4.9
182	22.9	19.0	—17.0
252	25.5	20.6	—19.1
322	26.0	19.4	—25.4
392	26.6	19.2	—27.8
462	23.8	19.3	—18.9

treated mice weighed less than control mice.

Discussion. The above experiments show that a ground lithosperm diet will markedly decrease the incidence of mammary tumor formation in two tumor-susceptible strains of mice. However, they do not prove that this decrease is due necessarily to drug administration.

Treated mice weighed approximately 18 to 28% less than control animals during the tumor developing period and consumed in the neighborhood of 34% less fox chow per mouse. The caloric value of lithosperm is not known but presumedly the plant has some caloric value and therefore decreased, to some extent at least, this difference in food consumption between treated and control mice.

Tannenbaum and Visscher *et al.*(9,10) have shown that underfeeding and caloric restriction inhibit the formation of mammary tumors in mice, as well as other types of tumors. For example, Visscher *et al.*(9) decreased the incidence of mammary tumors in C_3H virgin female mice, of the same subline as used in the present experiment, from 67% to 0 by restricting caloric intake by one-third. However their restricted mice weighed only 12 to 14 g as compared with 30 to 32 g for the *ad libitum* mice, a decrease of approximately 58%. Tannenbaum(10c) found that decreasing the daily caloric intake from 11.7 to 8.5 (a 27.3% decrease) in C_3H virgin females decreased body weight from 28 to 20 g (a 28.5% decrease) and lowered the incidence of mammary tumors from 57% to 0. The results with lithosperm on body weight and incidence of tumor formation in C_3H mice are in fair agreement with those of Tannenbaum with caloric restriction. The actual caloric intake of mice in the present study is not known.

Huseby *et al.*(11) think that caloric restriction reduces the incidence of mammary tumors in mice by producing a pituitary insufficiency, with resultant decreased ovarian secretion and a relative refractoriness of the mammary gland to estrogenic stimulation. Lithosperm has been shown to decrease the formation and/or action of gonadotropic hormones(5,6,8). In the present experiments it is impossible to determine the part played by lithosperm and that due to possible caloric restriction in decreasing the incidence of tumor formation. When the active principle of lithosperm is isolated, further studies along this line should be of value.

Summary. Ground *Lithospermum ruderale*, administered by drug-diet methods, lowered the incidence of mammary tumors from 58.1% to 2.8% in C_3H mice and from 96.6% to 21.9% in ZD_8F_1 mice. The exact roles played by the drug and by possible diet restriction were not determined.

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Received October 30, 1950. P.S.E.B.M., 1950, v75.

Influence of ACTH and Cortisone upon Alteration in Capillary Permeability Induced by Hyaluronidase in Rats.[*] (18341)

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The scope and nature of the physiological processes which come under the influence of the adrenal cortex is only beginning to be appreciated. Alterations induced in the animal body by the administration of adrenal cortical steroids are widespread and are obtained in many tissues(1). It has been suggested(2-4) that the plasticity of the ground substance of connective tissue may be, in part, under the control of these steroids. The permeability of connective tissues and membranes to dyes, particulates and fluids is increased following local application of hyaluronidase preparations(5,6,7). Intravenous administration of the enzyme produces an increase in capillary permeability(8,9). Following adrenal stimulation (produced by ACTH, epinephrine or stress conditions) or the administration of Compound E or adrenal cortical extract, the alterations in connective tissue and membrane permeability resulting from hyaluronidase are reported to be suppressed(3,5,10).

The present investigation demonstrates the inhibitory effect of ACTH and cortisone acetate on the increase in capillary permeability induced by the intravenous administration of a hyaluronidase preparation.

Materials and Methods. Female Sprague-Dawley rats weighing approximately 184 g were fed Purina chow and water *ad lib.* Weights were noted at the start of the experimental period and at the time of the hyaluronidase injection. Two mg of Evans blue in 0.2 or 0.5 ml of saline were administered intravenously by tail vein. One-half or one ml of a saline solution of bull testis hyaluronidase, assaying 184 units[†] per mg, and containing 2000 units, was given by tail vein immediately following the dye. Control animals received an equal volume of saline. The concentration of Evans blue in the blood, total serum protein, and hematocrit were de-

TABLE I. Influence of ACTH and Cortisone Upon Serum Dye Concentration, Serum Protein Concentration, and Hematocrit Measured 30 Min. After Inj. of a Hyaluronidase Preparation.

	No treatment*	ACTH	Cortisone
Evans blue conc. corrected for body wt/ml serum			
Saline	205 ± 4.2	194 ± 4.9	199 ± 4.1
Hyaluronidase	155 ± 3.3	177 ± 3.5	168 ± 5.3
% inhibition	—	67 ($p < .001$)†	40 ($p < .05$)
Serum protein concentration g %			
Saline	6.40 ± .11	6.20 ± .2	6.78 ± .1
Hyaluronidase	5.32 ± .19	5.84 ± .1	6.12 ± .2
% inhibition	—	67 ($p < .05$)	40 ($p > .1$)
Hematocrit—%			
Saline	48.6 ± 1.3	52.2 ± 1.8	51.8 ± 1.5
Hyaluronidase	57.2 ± 0.9	51.3 ± 2.3	52.4 ± 2.1
% inhibition	—	110 ($p < .01$)	93 ($p < .01$)

* The values are the means and standard errors for each treatment group. Each group consisted of 10 rats. The experiment was done in 2 parts, 4 animals per group in the first part and 6 animals per group in the second part.

† P values are for the order of significance of the difference in hyaluronidase effect between treated and untreated animals.

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TABLE II. Influence of Duration of Treatment with Cortisone Acetate Upon Disappearance of Evans Blue from the Blood of Rats Injected with Hyaluronidase. Evans blue concentration corrected for body weight per ml of blood.

	12 hr		24 hr		48 hr		96 hr	
	Control	Cortisone acetate[†]	Control	Cortisone acetate	Control	Cortisone acetate	Control	Cortisone acetate
Saline	126 ± 1.4	127 ± 2.7[*]	126 ± 2.2	122 ± 0.5	132 ± 4.4	131 ± 1.9	121 ± 3.1	122 ± 0.8
Hyaluronidase	90 ± 7.0	92 ± 5.4	75 ± 3.7	85 ± 7.6	82 ± 3.5	101 ± 5.0	97 ± 2.4	113 ± 3.4
% inhibition		3($p>.9$)		26($p>.1$)		40($p<.05$)		64($p<.01$)

[*] This group contained 4 animals; all other groups consisted of 5 animals.

[†] 5 mg per animal per day.

termined 30 minutes following the administration of the enzyme by methods previously described(12). In the experiments shown in Table I, the dye concentration was measured in the serum; whole blood was used in subsequent experiments. The ACTH[‡] was administered subcutaneously in 2 daily doses of 2.5 mg per 0.5 ml of an 0.85% saline solution. Cortisone acetate was given subcutaneously as a suspension diluted with saline so that each animal received 2.5 mg twice a day except as indicated in Table II. Evans blue concentration following the injection of a constant quantity of dye varies inversely as the plasma or blood volume and blood volume varies as the body weight(13). In order to compare the serum or blood dye concentrations in animals of differing weights a correction was introduced. Dye concentration in μg per ml of serum or blood was multiplied by the body weight of the animal taken at the time of the test. The values were all divided by 200 g to yield concentration of dye per ml of serum or blood for a 200 g rat. These values are recorded in the tables.

Results. The experiment in Table I was designed to test whether ACTH or cortisone acetate would inhibit the increase in capillary permeability induced by testicular extract. One group of animals was pretreated for 5 days with ACTH, another with cortisone acetate, and a third received no treatment. Each group was then divided in half. One half of the animals received saline and Evans blue intravenously, the other half received hyaluronidase and Evans blue. Table I demonstrates that both ACTH and cortisone acetate inhibited the effects of the hyaluronidase. The inhibition of the change in the 3 indices of enzyme action studied (dye concentration, serum protein, and hematocrit) was statistically significant in all but the effect of cortisone upon the serum protein change.

The experiment shown in Table II was evolved to determine how soon the inhibitory action of cortisone acetate becomes manifest. Groups of 5 animals each were injected subcutaneously twice daily with the steroid suspension while control animals were treated with saline. Twelve, 24, 48, and 96 hours following initiation of therapy, rates of Evans blue disappearance were tested in groups of animals after injection of enzyme solution or saline. Although the inhibition of the effect of testicular extract appeared 24 hours following initiation of therapy, a statistically significant reduction did not occur until 48 hours had elapsed.

A single large dose of 20 mg of cortisone acetate, as obtained, in 0.8 ml of the commercial diluent[§] was administered subcutaneously to a group of 10 animals. Controls received an equal volume of the diluent subcutaneously. Twelve hours later they were tested as in the previous experiment. No inhibition was demonstrable.

Discussion. The exact nature of the action

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[‡] ACTH was obtained through the courtesy of Dr. Edwin E. Hays of the Armour Laboratories. The dosage refers to LA-1-A standard.

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[§] Cortisone acetate suspension was purchased from Merck and Co.

of intravenously administered hyaluronidase in the rat is unknown. The increased rate of disappearance of Evans blue from the circulation, the decrease in serum protein concentration, and the increased hematocrit following injection of the enzyme indicate an increase in capillary permeability. Histologic examination of the grossly edematous areas in these animals(14,15) does not show rupture of the capillary wall as seen by Zweifach and Chambers(16).

Present knowledge(17,18) of the *in vitro* action of partially purified testicular extracts makes it reasonable to assume that the enzyme acts upon such polysaccharides as hyaluronic acid and chondroitin sulfate. If these polysaccharides are components of the intercellular substance of the capillary, the present work suggests that the intercellular substance becomes resistant to the action of testicular hyaluronidase following administration of ACTH and cortisone. To the extent that there is increased resistance of the capillary to the action of testicular enzyme our data agree with the reports of others who found that local and distant administration of adrenal cortical extract inhibited the spreading and permeability increasing effect of testicular extract in skin(3,19) and synovial membranes(4).

An immediate effect due to local application of adrenal substances and the rapid emergence of an effect following distant administration(3,4,19) suggest a direct action of the compounds on connective tissue. Inhibition *in vitro* of the action of hyaluronidase on membrane permeability(5) is also consistent with the hypothesis of a direct action. In contrast, our data show no significant inhibition until 48 hours of treatment had been given. From the results of this investigation one may postulate an intermediate step between the administration of cortisone and its action on the tissue. The following possibilities exist: 1) cortisone acetate is not the active substance and must be modified by some cellular process to another compound which acts directly on the tissue or the enzyme, or 2) cortisone affects the cells of the mesenchyme which in turn alter the intercellular substance. Winter and Flataker present evidence indicating that cortisone acetate is not the locally active compound. Such activity, however, was found to be present in adrenal cortical extract(20). That cortisone acetate has an effect on mesenchymal cells has been demonstrated(21,22).

Summary. Intravenous administration of 2000 units of partially purified testicular hyaluronidase in rats induced an increased rate of disappearance of Evans blue, a decreased concentration of serum protein, and an increased hematocrit. These manifestations of an increased capillary permeability due to the action of the enzyme preparation were largely inhibited by 5 days pretreatment with subcutaneous administration (5 mg per day) of either ACTH or cortisone acetate. A minimum of 24 to 48 hours of pretreatment with cortisone acetate was required for a discernible inhibition of the hyaluronidase effect. The evidence suggests an intermediate step between cortisone acetate and its action on connective tissue.

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Received November 8, 1950. P.S.E.B.M., 1950, v75.

Effects of Feeding Autoclaved Pancreas to Depancreatized and Duct-Ligated Dogs.* (18342)

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Dragstedt, van Prohaska, and Harms(1) concluded as a result of experimental studies that the beneficial effect of feeding fresh pancreas to depancreatized dogs was due to the presence in pancreas of a specific substance aside from lecithin or choline, and for which they suggested the name "lipocaic," believing it to be a hormone concerned with the utilization of fat. They found that liver and brain tissue, which contained lecithin and choline in amounts roughly equivalent to that present in pancreas, were entirely ineffective. Fat-free pancreatic extracts were prepared from which most of the choline originally present in the pancreas had been removed, and these were found to be effective, while other extracts containing the fat of the pancreas and most of its choline and lecithin were ineffective. Recently, Chaikoff and Entenman(2) have come to the conclusion that the anti-fatty liver factor of pancreas is a proteolytic enzyme, which, by its digestive action in the intestinal tract, liberates the methionine of protein and possibly other lipotropic agents for more effective action. It is proposed that in the absence of pancreatic juice from the intestinal tract, a disturbance in the digestion of proteins results, which in effect deprives the body of lipotropic agents present in the diet, which fail to be absorbed under these conditions. This thesis is supported by their finding that the oral administration of fresh pancreatic juice exerts a beneficial effect in preventing the development of fatty livers in insulin-treated, depancreatized dogs and in animals whose pancreatic ducts have been ligated. It is also supported by their observation that diets containing hydrolyzed casein were more effective in preventing fatty livers than similar diets containing equivalent amounts of unhydrolyzed casein, and their demonstration that pancreatic extracts prepared by them, and which were found to be effective in the treatment of depancreatized dogs, are heat labile. In a recent study, Montgomery, Entenman, Chaikoff and Fineberg(3) have reported that the oral administration of as little as 5 mg of crystalline trypsin to each lean meat meal fed to insulin-treated, depancreatized dogs completely prevented the development of fatty livers in these animals. Best(4) commented on the work of Palmer in his laboratory, who apparently found that the active principle in extracts of pancreas prepared by the method of Entenman and Chaikoff was trypsinogen and chymotrypsin. Best(5) has elaborated his view that the beneficial effect of pancreas feeding to depancreatized dogs can be accounted for on the basis of its content of known lipotropic agents, such as choline, methionine, or vitamin B_{12}, which may potentiate or spare choline.

These newer developments have prompted the present report of observations begun many years ago and which have recently been confirmed and extended. They demonstrate that beef pancreas, autoclaved at 15 lb pressure for from 15 to 30 minutes, continues to exert its beneficial effect in insulin-treated, depancreatized dogs and in dogs whose pancreases have become extensively degenerated as a result of ligation of the pancreatic ducts. Liver, brain, and salivary glands similarly treated exert no such beneficial effect. The

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* This work has been aided by a grant from the Otho S. A. Sprague Memorial Institute.

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TABLE I. Effect of Feeding 200 g Autoclaved Pancreas Daily to Depancreatized Dog, D977.

Date 1948	Remarks	Wt, kg	Serum lipids mg %	Alkaline phosphatase of serum units	Liver fat	Insulin units
4/7	Total pancreatectomy	14.7	747	8.5	0	—
5/11	No supplements	12.4	504	68.5	+++	78
8/30	” ”	14.2	536	58.0	+++	88
9/29	” ”	13.1	340	59.1	++	80
3/16/49	” ”	10.1	380	123.4	++	16
3/17	200 g autoclaved pancreas daily					
3/30	” ” ” ”	11.7	694	41.7		36
4/13	” ” ” ”	12.0	727	24.5		40
4/27	” ” ” ”	13.7	925	18.8	+	52
5/5	No supplements					
5/11	” ”	13.2	565	51.9	++	30
6/8	” ”	11.7	573	89.0	+++	34
7/7	” ”	12.2	436	125.4	+++	68
7/19	Died				21.1%	

development of lipocaic insufficiency in depancreatized dogs, whose diabetes is controlled and glycosuria prevented by the adequate administration of insulin, is manifested by the following signs and symptoms: a progressive decrease in body weight and disappearance of body fat, a progressive increase in the alkaline phosphatase of the serum, a decrease in the amount of insulin required to prevent glycosuria, a progressive decrease in the concentration of the blood lipids, the development of fatty infiltration of the liver with impairment of liver function, and an increase in sensitivity to insulin so that hypoglycemia convulsions are difficult to prevent. Some or all of these findings appear in about 90 percent of insulin-treated depancreatized dogs and in the majority of animals whose ducts have been ligated and the pancreatic parenchyma destroyed.

Fresh pancreas, liver, brain, and salivary glands of beef were ground, spread in pans, and autoclaved at 15 pounds pressure for 15 to 30 minutes. This treatment sterilized the tissues and inactivated the enzymes present as indicated by the fact that bits of pancreas placed in tubes of sterile salt solution and incubated for several days at 37°C displayed no evidence of digestion or bacterial growth. Pancreatectomized dogs and animals whose pancreatic ducts had been ligated, divided, and omentum interposed between the pancreas and duodenum, were used to determine if feeding the autoclaved tissues could prevent the onset of lipocaic insufficiency or reverse it if already developed. After recovery from surgery the animals were fed daily 800 g of horse meat, 50 g bread, 25 g cane sugar, 400 cc milk, 25 g suet, 30 g bone meal, and 5 g of brewer's yeast. During the period of test 200 g of autoclaved tissue were substituted daily for an equal amount of horse meat. The animals were weighed daily and the urinary sugar determined. Regular insulin was given in amounts adequate to prevent all but a minimum glycosuria. Needle biopsies of the liver were secured at two week intervals, and on these occasions determinations were made of the total serum lipids(6), the serum alkaline phosphatase, serum proteins, hemoglobin, and red corpuscles. Four depancreatized dogs with well developed findings of lipocaic insufficiency were completely relieved by feeding 200 g autoclaved pancreas daily for 4 to 8 weeks. Two depancreatized dogs were protected from lipocaic insufficiency by feeding autoclaved pancreas for 6 and 8 weeks, and when the pancreas was discontinued the insufficiency developed. Table I illustrates the curative effect of feeding autoclaved pancreas and the return of lipocaic insufficiency when the pancreas was stopped.

Eight dogs with ligated pancreatic ducts developed lipocaic deficiency, and in all of them the deficiency was entirely relieved by feeding autoclaved pancreas. Table II illustrates the curative effect of feeding autoclaved

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TABLE II. Effect of Feeding Autoclaved Pancreas and Autoclaved Salivary Glands to Dog D-77 with Ligated Pancreatic Ducts.

Date	Remarks	Wt, kg	Serum lipids mg %	Serum alkaline phosphatase units	Liver fat	Insulin units
3/25/49	Ligation of pancreatic ducts	10.9	730	8.4	0	0
3/26	200 g raw pancreas daily					
5/11	" " " "	10.9	750	6.4	0	2
5/12	No supplement fed					
5/25	" " "	10.1	505	14.9		0
6/22	" " "	9.2	313	43.4	++	0
7/20	" " "	7.4	315	66.8	+++	0
7/23	10 g defatted pancreas powder daily					
8/17	" " " " "	8.2	660	4.0	+++	0
10/5	" " " " "	9.0	702	4.5	++	0
11/12	No supplements					
11/29	" "	8.8	535	10.0	++	0
1/ 5/50	" "	7.1	220	14.5	+++	0
1/31	" "	6.3	137	28.1	++++	0
2/10	200 g autoclaved pancreas daily					
2/28	" " " "	6.2	702	4.7	++++	0
3/28	" " " "	8.0	873	6.9	++++	4
4/25	" " " "	9.4	753	7.5	+	8
5/31	" " " "	10.2	778	5.4	++	2
7/18	" " " "	10.5	905	1.2	0	2
7/26	200 g autoclaved salivary glands daily					
8/ 1	" " " " "	10.4	670	6.7	0	2
8/14	" " " " "	9.8	337	4.6		0
8/28	" " " " "	9.4	410	3.6	+++	0
9/25	" " " " "	8.9	238	17.8	+++	0
10/ 9	" " " " "	8.4	270	17.3	+++	0

TABLE III. Effect of Feeding 200 g Autoclaved Salivary Glands and Autoclaved Liver to Depancreatized Dog No. D-104.

Date	Remarks	Wt, kg	Serum lipids mg %	Serum alkaline phosphatase units	Liver fat	Insulin units
6/ 3/49	Pancreatectomy	19.8	790	2.8	0	0
6/ 8	200 g raw pancreas daily	19.7	925	5.6	0	30
6/22	" " " "	19.0	988	16.3	0	74
6/29	200 g autoclaved salivary glands					
7/ 7	" " " "	18.0	840	32.9	0	60
8/ 3	" " " "	16.9	780	16.6	0	44
8/17	" " " "	16.7	663	38.1	0	58
8/31	" " " "	15.9	565	40.9	+++	42
10/ 5	" " " "	15.4	548	44.9	++	78
1/31/50	" " " "	15.5	507	16.4	++	64
7/18	" " " "	15.7	770	10.7	++	48
7/26	200 g autoclaved liver daily					
8/14	" " " "	15.2	720	12.0	++	48
8/28	" " " "	15.3	545	24.0	+++	52
9/11	" " " "	15.0	463	29.5	+++	52

pancreas to one of these animals and the return of lipocaic deficiency when autoclaved salivary gland was substituted for the pancreas.

Autoclaved salivary gland was fed to 7 depancreatized dogs without any effect on lipocaic deficiency in six and a questionable beneficial effect on the seventh. It was fed to 2 dogs with ligated pancreatic ducts with lipocaic insufficiency but had no beneficial effect. Table III illustrates the ineffectiveness of autoclaved salivary gland and liver in preventing the development of lipocaic insufficiency in a depancreatized dog.

Autoclaved liver was fed to 4 depancreatized dogs with a negative effect in 2 and a questionable effect in 2. Autoclaved brain was fed to 4 depancreatized dogs without beneficial effect in 2 and questionable effect in 2.

It should be emphasized that the onset of lipocaic deficiency in depancreatized dogs varies greatly in different animals so that preventive experiments are less valuable than curative.

The demonstration in these experiments that the oral administration of autoclaved pancreas is effective in preventing and relieving the syndrome of lipocaic insufficiency in insulin treated depancreatized and duct ligated dogs indicates that the active principle, lipocaic, is relatively heat resistant and is accordingly not trypsin or any of the known pancreatic enzymes. Furthermore, the observation that liver, brain, and salivary gland similarly treated are ineffective indicates that the active principle in pancreas is not one of the known lipotropic agents, such as choline, methionine, or inositol, or a combination of them, since these substances are apparently widespread in animal tissues and are present in liver, brain, and salivary glands in amounts roughly equivalent to that in pancreas. The conclusion seems inevitable that lipocaic is a specific substance present in pancreas but not in other organs, and which is effective on oral administration in relieving the residual deficiency in insulin treated depancreatized dogs. It was largely this conclusion which led us to suggest that lipocaic is a pancreatic hormone although it was appreciated that its mode of action was unknown.

In the first paper(1) is was suggested that lipocaic played a role in the transport and further utilization of fat and that choline might play some important intermediary role in this function. Both parathyroid extracts and large doses of calcium lactate have been found to be effective in relieving parathyroid deficiency. Perhaps in a somewhat analogous manner lipocaic and large doses of choline (2 to 2.5 g per day) are effective in relieving the deficiency in depancreatized dogs. It can be postulated that in normal animals the supply of lipotropic materials in the diet suffices for normal fat utilization in the liver when aided or potentiated by lipocaic from the pancreas. In the absence of lipocaic the amount of lipotropic material in the usual diet is inadequate and fatty infiltration of the liver results. The work of Chaikoff and his associates indicates that failure of pancreatic juice to reach the intestines makes the supply of lipotropic material in the food less available. Pancreatectomy thus operates in 2 ways to produce the disturbance: namely through the removal of lipocaic and through a reduction in the amount of available lipotropic material. The oral administration of fresh pancreas likewise operates in two ways by providing both lipocaic and pancreatic enzymes. This is supported by the observation we have made many times that, although effective, autoclaved pancreas is not as good as a similar quantity of the fresh gland. When diets containing minimum amounts of fat are fed, as in the experiments of Chaikoff and Entenman, restoration of pancreatic digestion alone apparently may suffice for a time at least to correct the deficiency. However, in our experience, when diets rich in fat were given, even large amounts of active pancreatic juice were ineffective.

Conclusions. 1. The daily oral administration of 200 g of beef pancreas autoclaved at 15 lb for 15 to 30 minutes was found to be effective in relieving and in preventing fatty infiltration of the liver and other manifestations of lipocaic deficiency in 6 depancreatized and 8 dogs with ligated pancreatic ducts. 2. Autoclaved liver, brain, and salivary glands were found to be ineffective under similar conditions. 3. There is a specific heat resistant substance in pancreas, lipocaic, not found in other tissues, which is effective on oral administration in relieving the residual deficiency in insulin treated depancreatized dogs.

Received November 8, 1950. P.S.E.B.M., 1950, v75.

Shortening of Bleeding Time by a Water-soluble Adrenochrome Derivative.* (18343)

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Oxidation and other chemical modifications of the epinephrine molecule affect its various physiological properties in varying degree and sense. Oxidative ring closure which leads from epinephrine to adrenochrome converts a pressor into its opposite, an antipressor substance(1). The hemostatic effect of epinephrine is altered in degree, but not in sense by the same chemical change(2). The property of adrenochrome of shortening the bleeding time is shared by adrenalone, a less profoundly modified oxidation product of epinephrine(3).

Because of the limited solubility and of the instability of adrenochrome in solution, one of us has prepared a series of highly water-soluble adrenochrome derivatives which will be described elsewhere(4). The observations of Derouaux that adrenochrome shortens the bleeding time in rabbits and subsequent reports(5) that similar effects could be obtained with the monoxime, monosemicarbazone and mono-dinitro-phenylhydrazone of adrenochrome suggested a study of the hemostatic effect of a compound representing our series of water-soluble adrenochrome derivatives. We selected the trimethylammoniumacethydrazone hydrochloride of L-adrenochrome (T.A.L.). Observation of a shortened bleeding time upon administration of this compound would indicate that the chemical modification of the parent substance adrenochrome in this type of derivative does not impair one of its physiological effects.

Material and methods. Trimethylammoniumacethydrazone hydrochloride of L-adrenochrome is a red substance melting with decomposition at 160°; it is extremely soluble in water. The finely ground substance was mixed with 4% of its weight of anhydrous disodium phosphate and distributed in the desired dosage, usually 10 mg, in sterile ampules. The contents were dissolved in sterile distilled water immediately before use, giving a solution of pH 6.5. The bleeding time was determined by the method of Ivy (6). A blood pressure cuff is placed around the arm and inflated to 40 mm Hg pressure. The cleaned skin of the forearm is pricked with an automatic lancet. The drop of blood which collects at this site is removed every 10 seconds by blotting paper; this permits the determination of the bleeding time within 5 to 10 seconds.

Results. In a group of preliminary tests, amounts up to 2 mg were administered by Dr. S. E. Moolten at St. Peter's Hospital in New Brunswick, N. J., to a number of patients by intramuscular injection and in a few cases *per os*. The bleeding time was determined before and about 1 hour after the medication. In two-thirds of the cases the bleeding time was diminished after administration of the compound and neither local reactions nor any other untoward symptoms were seen. On the basis of these preliminary observations we felt encouraged to study a larger series. A group of 50 consecutively admitted children, between the ages of 2 and 16, undergoing tonsillectomy and adenoidectomy were studied with the proper controls to exclude the effects of simultaneous surgical procedures. Of the 50 subjects two-thirds were injected

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* We are indebted to the Life Insurance Medical Research Fund for financial support.

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intramuscularly with 10 mg of T.A.L. dissolved in 1 ml of water, while one out of 3 served as control. The injection was administered immediately before removal of the patient to the operating room. In view of the nature of the operation no clinically noticeable effects were to be expected. No reactions of any sort were noted in the children receiving the medication. There was no unusual change in the pulse. The amount of bleeding in these children during the course of the tonsillectomy and the adenoidectomy appeared about the same as in the control group. No blanching or other changes in the mucous membranes of the pharynx were noted. There was no post-operative hemorrhage in either control or the treated group. There were no local reactions at the site of the injection.

The bleeding time was again taken after the patient had returned from the operating room and recovered from the anesthesia. The time interval between the two determinations varied from 3½ to 6 hrs and averaged 4 hrs 35 min for the treated cases and 4 hrs 30 min for the controls. Whether a patient had received medication or not, remained unknown to the person who measured the bleeding time.

As shown in Fig. 1 the bleeding time was found reduced in 25 out of 32 cases to values as low as 22% of the original bleeding time. In 6 cases there was no difference between the bleeding time before and after administration of T.A.L. and in one case an insignificant lengthening. The average shortening of the bleeding time was 38% for all cases, but 49% for those 25 cases which showed any response at all. It is noteworthy that the pre-injection bleeding time of the responsive group averaged 4 min (238 sec) as against 141 sec for the 7 non-responsive individuals. This indicates that the hemostatic mechanism, upon which T.A.L. acts, hardly permits of further enhancement in cases with an initial bleeding time of 2 min. No correlation was observed between the response and the age and sex of the patients.

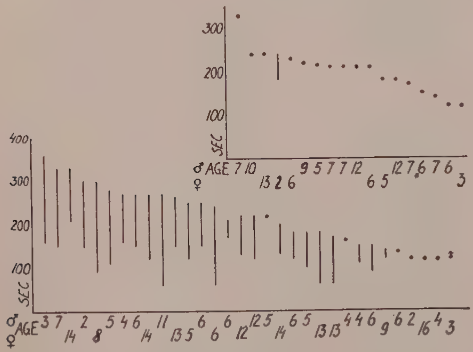

FIG. 1.

Shortening of bleeding time in a group of 32 children upon administration of 10 mg T.A.L. The vertical lines connect the point which indicates pre-injection bleeding time with the point giving the bleeding time after an average period of 4½ hr following the injection. The bleeding time in non-responsive cases is indicated by dots.

In the control group of 18 non-treated children with a similar distribution of age and sex, the bleeding time varied individually from 120 to 330 sec (See inset, Fig. 1). The median bleeding time of this group was identical with that in the injected group, namely 210 sec. The average bleeding time of the controls was 201 sec in satisfactory agreement with 218 sec, the overall average of the injected group. The bleeding time of the control cases remained remarkably constant before and after the surgical intervention; in 17 cases the bleeding time was identical within 5 sec as measured before and after the operation. In only one control case, a drop of bleeding time from 240 to 180 sec was encountered. It was noted in several cases that the bleeding time had returned to its original value when they were retested on the third day after an effective injection. Likewise the bleeding time of several control cases proved identical when retested 3 days later.

Discussion. The mechanism of the hemostatic effects of sympatomimetic compounds and of their oxidation products has been studied and discussed in numerous publications by Roskam of Liège and his school(2,3,5). Other physiological effects of these products have been investigated by Bacq(7). Oster(8) has dealt with the mechanism of their antipressor

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action. The therapeutic exploitation of these various pharmacological properties requires the appropriate form for administration. New compounds, synthesized by modification of chemical functional groups with a view to increased stability and solubility, must be compared with the parent substance in respect to the perseverance of the desired activity. In the present instance, we have converted adrenochrome into the stable and extremely water-soluble betaine hydrazone T.A.L. The clinical tests which we report show that the formation of the derivative has not destroyed the hemostatic effects of adrenochrome. These results, in addition to their practical interest *per se,* may serve as a guide in the study of the metabolic fate of the compounds; moreover, they point to the localization of the hemostatic effect in that part of the molecule not affected by the synthetic chemical modification.

Summary. Trimethylammoniumacethydrazone hydrochloride of L-adrenochrome (T.A.L.) is a potent hemostatic agent. It shares this property with the parent substance adrenochrome, but surpasses it by stability and solubility in aqueous solution. It may be injected intramuscularly in children in amounts of 10 mg without producing local reactions. A single injection of this amount shortens the bleeding time, as measured by Ivy's method, by an average of 38% in 32 subjects; except for 7 non-responsive cases with short pre-injection bleeding time, the mean bleeding time in the remaining 25 cases is cut in half. Only individuals with a short bleeding time fail to respond. Control tests show that the concomitant surgical intervention has no influence on bleeding time.

We are grateful to Dr. Sylvan E. Moolten, Director of Laboratories, St. Peter's Hospital, New Brunswick, N. J., for the preliminary clinical tests and for various suggestions, to Dr. Rudolph Kramer, Otolaryngologist to the Mount Sinai Hospital, for the facilities of his Service, to Mr. John Austin for the preparation of the compound and to Miss Aina Birnbaum for measuring bleeding times.

Received November 10, 1950. P.S.E.B.M., 1950, v75.

Effect of Maleic Acid on Renal Function. (18344)

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(Introduced by A. B. Gutman.)

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As part of an investigation of the capacity of certain organic acids to serve as effective buffers for the excretion of titratable acid, maleic acid was selected since its pK (about 6.5) is such as to be theoretically well suited to this function. It was observed, however, that when maleic acid was administered to an acidotic dog, the urine became alkaline despite the fact that the animal was in severe acidosis (plasma pH 7.0, CO_2 content 9.5 mM/1). Further investigations of the effects of maleate on renal function are the basis of the present report.

Methods and material. Trained, unanesthetized female dogs in the post-absorptive state were used in all experiments. Acidosis was induced by the administration of 5 g of NH_4Cl eighteen hours before some of the experiments and in others by the inclusion of dilute HCl in the infusion fluid. The technics and chemical methods used have been described in earlier publications from this laboratory(1,2). Maleate, generally at a dosage of 40 mg (0.35 mM) per kilo, was administered intravenously as a single dose in all experiments. The pH of the injected material

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TABLE I. Effect of Maleate on Acidification of the Urine. Dog F, Weight 16 kg.

Time, min.	Creatinine clearance, ml/min.	Plasma CO_2 content mM/l	Urine pH	Urine Titratable† acidity, μEq./min.
−66	Start infusion: creatinine 0.4%, NaCl 50 mEq./l, Na_2HPO_4—NaH_2PO_4 (pH 5.8) 70 mM/l at 6 ml/min.			
−58 to −56	HCl (0.1 N) 50 ml, i.v.			
0- 20	68.5	15.5	5.85	136
20- 41	73.0		6.00	186
41- 56	76.7	16.1	6.00	203
58	Maleic acid—40 mg/k, i.v.			
56- 76	84.0	—	6.60	127
76- 96	66.5	13.9	7.22	2
96-116	57.0	—	7.38	0
116-137	63.2	10.6*	7.38	0

* Arterial plasma pH 7.26.
†All urines titrated to pH 7.40.

was usually brought to about 6.0 by the addition of $NaHCO_3$ to a solution of maleic acid.

Results. The most striking effect of maleate was on the acidity of the urine. In every instance, there was a sharp rise in the urinary pH observable in specimens obtained 15-20 minutes after injection of the maleate and persisting for a period of 3-4 hours after the single dose. The extent of the rise in urine pH was dependent in part on the severity of the acidosis. When the bicarbonate concentration of the plasma was 10 mEq/l or more, the pH of the urine generally rose to or above that of the plasma. In more severe acidosis, the pH of the urine increased, but frequently remained lower than that of the plasma. When the urine was alkaline before the injection of maleate, there was little or no further increase in urinary pH, but there was a further rise in the excretion of bicarbonate. An experiment illustrating the effect of maleate on the urine pH and titratable acidity is presented in Table I. In this experiment, the dog was rendered acidotic by the intravenous administration of HCl while urinary buffer was provided by the infusion of phosphate. The characteristic effect on urinary acidity in the face of moderately severe acidosis is shown.

Glomerular filtration was variably affected by maleate in the dosage used. In 18 of the 34 experiments, the creatinine clearance remained essentially unchanged or increased slightly. In 6, there was a drop of 10-20% and in 10 experiments, the creatinine clearance fell 20-50%. Nine of the 10 marked falls in filtration rate were observed in experiments in which glucose was also administered, for the measurement of glucose Tm (8 exp.) or as the diluent for the infusion (1 exp.).

Tubular capacity for the secretion of p-aminohippurate was measured in 5 experiments. In 4 of these, the Tm_{PAH} was found to be depressed an average of 15% (mean of all clearance periods after the maleate injection). In one additional experiment, the only one in which the PAH was administered in glucose solution, there was a fall of 55% in Tm_{PAH} along with a marked drop in creatinine clearance. An experiment showing the effect on PAH transport is shown in Table II, along with data to indicate the typical changes in electrolyte excretion following the administration of maleate.

The major effects of maleate on electrolyte excretion were those associated with impairment of the mechanism of acidification and bicarbonate reabsorption. Most of the increase in cation loss was due to the rise in sodium excretion; potassium excretion increased to a moderate extent.† No specific effect on the excretion of chloride was apparent. The excretion of endogenous phosphate

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† Potassium secretion did not appear to be inhibited in an experiment in which maleate was administered during the infusion of potassium salts.

TABLE II. Effect of Maleate on PAH Transport and Excretion of Electrolyte. Dog B, Weight 20 kg.

Time, min.	Urine flow, ml/min.	Creatinine clearance, ml/min.	Tm_{PAH} mg/min.	Plasma HCO_3^- mEq./l	Urine pH	Rate of excretion: Na^+ µEq./min.	K^+ µEq./min.	Cl^- µEq./min.	HCO_3^- µEq./min.	PAH^- µEq./min.	$H_2PO_4^-$ $HPO_4^=$ µM/min.
−33 to −31	Creatinine 1.75 g, PAH 2.0 g, i.v.										
−30	Start infusion creatinine 0.45%, PAH 1% in water at 6 ml/min.										
0- 17	0.9	77	12.6		5.20	85	93	0	0.3	246	0.9
17- 38	0.4	64	12.5	20.3	5.12	40	82	0	0.1	212	—
38- 63	0.9	69	11.3		5.09	24	89	0	0.1	222	0.6
67	Maleic acid 40 mg/kg, brought to pH 6.5 with $NaHCO_3$, i.v.										
63- 86	2.0	68	12.3		5.72	134	115	10	1.1	214	—
86-110	3.5	80	13.4	19.9	6.00	273	156	0	4.5	250	11.7
110-133	5.7	74	10.4	20.5	6.90	288	151	0	38	224	—
133-153	7.2	73	10.5	20.5	6.97	292	131	0	53	221	17.6
153-173	6.2	74	8.6	19.4	7.18	331	117	0	76	222	—
173-193	7.3	75	9.8	19.9	7.37	425	128	0	133	232	39.4

generally showed a moderate increase after the injection of maleate.[‡] There was a marked excess of measured cation over anion in the periods following the injection of maleate. Attempts to identify the anion responsible were unsuccessful. The total maleate injected was insufficient to account for the excess anion excretion and the titration curve of the urine did not suggest the presence of appreciable concentrations of maleate. Quantitative measurements showed that the change in anion excretion was not attributable to organic or inorganic phosphate, inorganic sulfate, citrate, or α-amino acids. Qualitative tests for dicarboxylic and keto acids were negative.

In nine experiments in which glucose Tm was measured, glucose reabsorption fell more or less markedly after maleate was administered. Interpretation of these results was made difficult, however, by the marked drop in the rate of glomerular filtration that almost invariably occurred. The decrease in glucose reabsorption was always greater, proportionately, than the change in creatinine clearance.

Toxicity. As is apparent from the effect on creatinine clearance of maleate during the infusion of glucose solutions, this combination of substances, for undetermined reasons, was considerably more toxic than maleate alone in the same dosage. Although animals given maleate alone showed transient impairment of renal function, all promptly recovered and showed no persistent impairment of health or renal function despite repeated dosage. Several of the dogs given glucose and maleate together died 2-3 days later and others showed marked elevation of the blood urea nitrogen and marked acidosis lasting for 1-2 weeks afterward. Post-mortem examination of animals that died showed diffuse necrosis of the convoluted tubules. No pathological studies were done on animals which did not receive both glucose and maleate as all such animals survived.

[‡] In experiments with infused phosphate, the calculated capacity to reabsorb phosphate decreased an average of 28%. In our hands, a stable reabsorptive capacity for phosphate has not, however, been demonstrable under other circumstances.

Effect on acid secretion by the frog gastric mucosa. The effect of maleate on acid secretion by the frog gastric mucosa was studied by the technic of Patterson and Stetten (3). Because of the buffering capacity of maleate in the physiological range of pH, it was not possible to measure the secretion of H^+ ions by the stomach into a solution containing appreciable amounts of maleate and the maleate was, therefore, added only to the chamber in contact with the external surface of the mucosa. No inhibition was evident with maleate concentrations of 100 mM/1. There may be some question of the penetration of the maleate to the region of acid formation under the circumstances of this experiment. NaF, 10 mM/1, was completely inhibitory when added under similar conditions.

Discussion. Maleate appears to exert an effect on acidification of the urine which is unique among substances whose action has been reported. Sulfanilamide impairs the excretion of titratable acid(4), but the effect is relatively slight and can be demonstrated only when acidosis is relatively mild. Cyanide (5) and tartrate(6) have been found to abolish urinary acidification but in these instances the effect is observed along with almost complete loss of renal tubular activity and a loss of the ability to excrete a urine with a pH different from that of the plasma. The latter is not the case with maleate since the capacity to excrete an alkaline urine is retained; urines with pH over 7.8 have been observed repeatedly. Although maleate appears to depress most of the renal functions studied to some extent, the effect on acidification is far greater than its other actions.

Maleate has been observed to exert an inhibitory action on a number of enzyme systems studied *in vitro*(7-11). It is not clear through which, if any, of these systems the effects described here are exerted.

Summary. Maleate administered to severely acidotic dogs at a dosage of 0.35 mM/kg abolishes the capacity to excrete an acid urine. Lesser effects on other renal functions are produced.

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Received November 10, 1950. P.S.E.B.M., 1950, v75.

Biosynthesis of Radioactive Allantoin.* (18345)

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Heinrich and Wilson(1) reported experiments dealing with the carbon precursors of the tissue purines of the rat. Since allantoin is one of the main end products of purine

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* Aided by a grant from the American Cancer Society administered by the Committee on Growth of the National Research Council. The C^{14} used in this investigation was obtained on allocation by the United States Atomic Energy Commission.

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metabolism in mammals, it was felt that the isolation of allantoin from urine following administration of radioactive precursors would provide a relatively simple method of studying purine metabolism. For this purpose, a simple procedure for the isolation of allantoin from rat urine was developed. $NaHC^{14}O_3$, $CH_3C^{14}OOH$ and $NH_2C^{14}H_2C^{14}OOH$ were administered to rats, allantoin was isolated from the urines, and the resulting products degraded chemically to determine the position of the isotope.[†]

Experimental. Organic syntheses. Sodium bicarbonate was made from C^{14} barium carbonate. Carboxyl C^{14} acetate was made by the Grignard reaction, and carboxyl C^{14} glycine from carboxyl labeled ethyl bromoacetate and potassium phthalimide(3). Doubly labeled glycine was prepared from doubly labeled acetic acid as previously described(3).

Isolation of allantoin. After rinsing each cage and funnel with distilled water, the diluted urines were combined and neutralized to approximately pH 7 with dilute sodium hydroxide. A 10% solution of $AgNO_3$ was added in excess, and the suspension chilled to 0°C. Cold 5% NH_4OH was added dropwise with stirring to pH 8-9 and the suspension was allowed to stand overnight in the cold room. After centrifugation, the precipitate was washed once with cold water, and suspended in 200 ml of water. The silver was precipitated with H_2S, the suspension centrifuged, and the supernatant aerated. A solution of 10% mercuric acetate in 20% sodium acetate was then added until the supernatant failed to become cloudy on further addition. After chilling for 2 hours, the suspension was centrifuged and the precipitate washed once by stirring with cold water. The precipitate was suspended in 200 ml of water and decomposed with H_2S. The mercury sulfide precipitate was removed by centrifugation, and, after aeration, the supernatant was concentrated *in vacuo* to dryness. The residue was then extracted with a few ml of hot water, filtered hot, and the solution chilled overnight. The precipitated crude allantoin was removed by centrifugation, dissolved in minimal hot water and decolorized with charcoal. Upon chilling, analytically pure allantoin was usually obtained. For radioactivity studies, the allantoin was invariably recrystallized several more times. The average yield from the pooled 24-hour urines of 5 rats was 40-60 mg. M.P. 216-217°. Total nitrogen (Kjeldahl) 35.2-35.6%; theory 35.4%.

Chemical degradation of allantoin. The method employed was that described by Buchanan, Sonne and Delluva(4). Under these conditions it was found that 80 mg of allantoin yielded 20-30 mg of glyoxylic acid and sufficient urea for radioactivity measurements.

Results. Labeled bicarbonate. One rat (wt. 220 g) received 440 mg of labeled sodium bicarbonate in divided doses hourly for 7 hours. A total of 680,000 counts/minute was administered. The allantoin recovered from the urine of this animal was free of radioactivity. This is to be expected if purines are converted to allantoin by the loss of carbon No. 6. It has been demonstrated that bicarbonate is incorporated preferentially into position 6 of uric acid of the pigeon(4), and guanine and adenine of the rat(1).

Labeled acetate. The 5 animals on this experiment received a total of 632,000 counts/minute over a period of 5 days (subcutaneous injection twice daily). On degradation of the allantoin from these animals no radioactivity could be demonstrated in the urea fraction representing carbons 2 and 8. A trace of radioactivity observed in the glyoxylic acid fraction representing carbons 4 and 5 is believed to be of no significance. Heinrich and Wilson have similarly demonstrated that the carboxyl carbon of acetate is not incorporated into positions 2 and 8 of the adenine and guanine isolated from the tissues of the rats used in this experiment(1). It, therefore, appears that acetate is not a precursor of positions 2 and 8 in the purines

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[†] Preliminary report of a portion of this work has appeared elsewhere(2).

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3. Sakami, W., Evans, W. E. and Gurin, S., *J. Am. Chem. Soc.*, 1947, v69, 1110.

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TABLE I. Radioactivity of Allantoin After Feeding Carboxyl Labeled Glycine.*

Days	Counts/min./mM of carbon
1	40
2	120
5	250
6	260
7	290
8	310
10	350

* 50 mg of glycine containing 10,700 counts/min./mM carbon fed daily.

TABLE II. Radioactivity of Biosynthetic Allantoin.

Individual carbon atoms of allantoinΔ	$NH_2CH_2C^{14}OOH$* C/min./mM C	$NH_2C^{14}H_2C^{14}OOH$† C/min./mM C
#2	0	15
#8	0	15
#4	410	85
#5	70	80

* See footnote to Table I.

† 18 mg of glycine containing 7900 c/m/mM of carbon fed daily for 5 days.

Δ The conventional numbering system of allantoin has been used. The individual samples were oxidized to CO_2 and analyzed in the form of $BaCO_3$. Measurements were made with a thin mica-window counter and the counts calculated for infinitely thick samples.

of the rat. Elwyn and Sprinson(5) have also obtained evidence that carboxyl-labeled acetate is not utilized by the pigeon for the synthesis of uric acid.

Carboxyl-labeled glycine. Table I shows the results obtained on allantoin isolated daily from the urine of 9 rats fed carboxyl-labeled glycine. Fifty mg of glycine (10,700 counts/min./mM of carbon) was fed daily to each animal over a 10-day period. A rapid incorporation of isotope into allantoin is apparent from the results obtained after the first day. Although there is a tendency to level off, the concentration of C^{14} in the allantoin was still increasing at the end of the experiment. In order to obtain sufficient compound for degradation, 40 mg of allantoin isolated from the second day's urine was combined with 42.5 mg of allantoin from the 5th day. This pooled specimen had a count of 170 counts/min./mM carbon. The degradation of the allantoin demonstrated that the carboxyl carbon of glycine was incorporated primarily into position 4 of allantoin (Table II). The radioactivity of carbon 5 is probably due to inherent small errors usually observed in the oxidation of glyoxylic acid semicarbazone(1).

Doubly-labeled glycine. The degradation of the allantoin from the urine of the rats fed 18 mg of doubly-labeled glycine daily for 5 days (total = 95,000 counts/min.) demonstrated that essentially equal amounts of isotope enter positions 4 and 5 of the allantoin molecule; carbon 4 originating from the carboxyl carbon and carbon 5 from the methylene carbon of glycine (Table II). The presence of isotope in small quantity in positions 2 and 8 is in agreement with the findings of Karlsson and Barker(6) who demonstrated the incorporation by pigeons of the methylene carbon of glycine into positions 2, 4, 5, and 8 of uric acid. This result is consistent with the evidence obtained by Sakami(7) that the methylene carbon of glycine is converted to "formate."

Summary. 1. A convenient method for the isolation of allantoin from the urine of rats has been developed. 2. The carbon of sodium bicarbonate is not incorporated in the urinary allantoin of rats. This result is in accord with the fact that bicarbonate is incorporated only into position 6 of purines. 3. The carboxyl carbon of acetate is not involved in the biosynthesis of allantoin in the rat. 4. The carboxyl carbon of glycine is incorporated into position 4 of allantoin and the methylene carbon into position 5.

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Received November 13, 1950. P.S.E.B.M., 1950, v75.

Physiological Regulation of Phosphopyruvate Formation from 3-phosphoglycerate in Rat Tissues.* (18346)

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Previous experiments in this laboratory(1) have shown that tissues of adult male rats convert 3-phosphoglyceric acid to phosphopyruvic acid at a higher rate than the corresponding tissues of female animals. This observation suggested a further investigation of the regulatory mechanisms of this coupled enzyme reaction (phosphoglyceromutase ⟶ enolase). The experiments here described demonstrate that castration and hypophysectomy as well as injection of diethyl stilbestrol, testosterone, cortisone, and adrenocorticotrophic pituitary extract (ACTH) markedly influence this phase of glycolysis.

Experimental. Castration was performed on both adult male and female white rats. Hypophysectomized adult male rats were obtained from the Hormone Assay Laboratories, Inc.† and were received one day after operation. Controls for this group came from the same source. The body weight of adult rats ranged between 300 and 350 g. It appeared to be of interest to determine the effect of age on the rate of phosphopyruvic acid formation; therefore, young male and female rats were used in the experimental and control groups in which the effect of diethyl stilbestrol, testosterone, cortisone, and ACTH was tested. The weight of these young animals was between 120 and 150 g. The enzyme activity of tissues of individual animals in an experimental group was found to agree within the limits of the standard error reported previously(1). Control and experimental groups consisted of 5 to 8 animals each. The difference between the average enzyme activities of control and experimental groups was considered significant when it exceeded at least twice the standard error of the controls. Diethyl stilbestrol, testosterone and cortisone were injected intraperitoneally in form of freshly prepared dilute ethanol (30%) solution in a volume of 0.1 ml, ACTH was suspended in saline, 1 ml containing 10 mg extract.

Results. The experimental data are summarized in Fig. 1, 2, and 3, each column representing the average enzyme activity of each

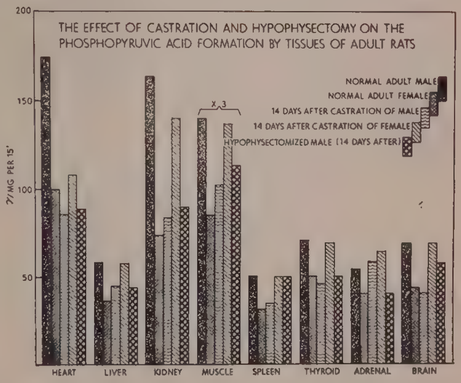

Fig. 1.

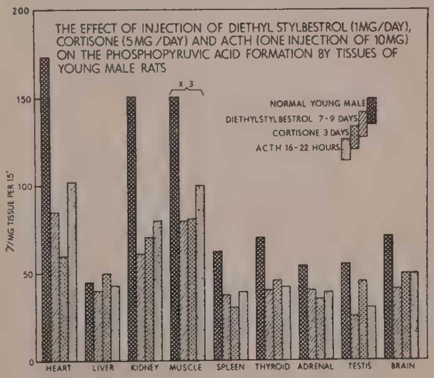

Fig. 2.

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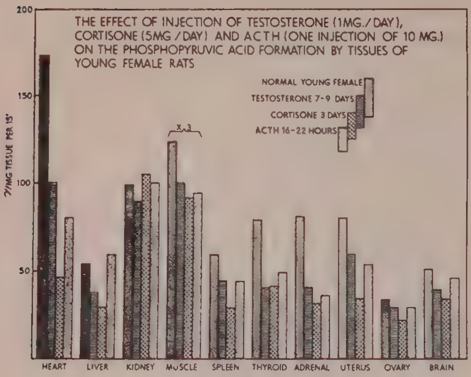

FIG. 3.

particular tissue. As early as 1 to 2 days after castration in both adult male and female rats, marked changes in enzyme activity could be observed. These changes persisted and were recorded 14 days after operation (Fig. 1).

In adult males castration caused a sharp decrease in the rate of phosphopyruvate formation, while removal of ovaries in females resulted in an increase. Heart, kidney, and muscle tissues exhibited the most pronounced changes, but this effect was also noticeable in other tissues. Hypophysectomy generally decreased the enzyme activity of tissues, but this effect was significant only 14 days after operation. Sham operations had no effect on the rate of phosphopyruvate formation.

In the course of enzyme assays of tissues of young rats it was noticed that the sex difference, observed on adult animals, was less pronounced in the young ones. Enzyme activities significantly higher in young males than in young females were observed only in kidney, muscle, and brain tissue. The enzyme activity of the adrenals of young females was somewhat higher than in young males. The rate of phosphopyruvate formation in the ovaries of young females was only about one-third of that in adult animals. Enzyme activities in young males was not significantly different from adult male animals.

The pharmacological action of diethyl stilbestrol on the rate of phosphopyruvate formation in young males, that of testosterone on young females, and the effect of cortisone and ACTH on enzyme activities of tissues of both sexes is summarized in Fig. 2 and 3. The action of these compounds was qualitatively similar; they caused a decrease in the rate of phosphopyruvate formation. Injection of diethyl stilbestrol (1 mg per day for 7 to 9 days) in young males resulted in a large decrease of enzyme activity in all organs, but less marked in the liver and adrenal. Testosterone injection of females (1 mg per day for 7 to 9 days) had a similar inhibitory effect, but this inhibition was small in the kidney. Cortisone (5 mg per day for 3 days) caused a marked diminution in phosphopyruvate formation, except in the liver of males, and kidney of females. The single injection of 10 mg ACTH resulted after 16 to 22 hours in a decrease of enzyme activity in all tissues, except in the liver of both sexes, and in the kidney and brain of females (Fig. 2 and 3).

Discussion. According to the observations reported in this paper, the phosphoglyceromutase-enolase reaction in tissue homogenates is a suitable test system for the study of hormones on enzyme reactions. At present it is difficult to visualize the exact mechanism of this hormonal regulation, since the injection of a hormone or the removal of an endocrine gland undoubtedly influences the whole regulatory system. Preliminary experiments *in vitro* with testosterone, dehydroandrosterone and desoxycorticosterone acetate showed that these compounds can activate or inhibit—depending on the amount of hormone—the phosphopyruvate formation in homogenates in amounts of 0.005 to 10.0 μg. Similar *in vitro* effects of sterole hormones were recorded by Dirschel and Hauptmann(2) on the glycolysis of mouse and rat liver slices and diaphragms. It is of interest that marked sex differences were found in tissue respiration of muscles of male and female specimens of *Periplaneta americana* by Barron and Tahmisian(3). Recently Copenhaver, Stafford and McShan reported(4) that the esterase activity of tissues of male rats was higher

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than of females, a finding comparable to the sex difference in the rate of phosphopyruvic acid formation(1) in rat tissues. It must be noted, however, that this sex difference in the rate of phosphopyruvic acid formation was not found in mice, indicating the influence of species on the regulation of enzyme reactions. Pertinent to the experiments reported is the effect of diethyl stilbestrol and castration on the metabolism of prostatic tissue, described by Barron and Huggins(5). The regulatory effect of hormones may partly be due to their effect on the synthesis of enzymes and partly to their interaction on the catalytic protein. This question is being further studied by comparison of *in vivo* and *in vitro* effects of pure hormones.

Summary. Castration of male white rats resulted in a decreased rate of phosphopyruvic acid formation from 3-phosphoglyceric acid, by dilute tissue homogenates. Ovariectomy caused an elevation of enzyme activity in the tissues of females. Enzyme activity of hypophysectomized rats decreased only 14 days after the operation. The effect of age on this enzyme reaction was also demonstrated. Tissues of young females had a higher enzyme activity than those of mature females, except the ovaries which were considerably lower than in the mature animals. The effect of age was not noticeable in young males. Injection of diethyl stilbestrol in young males or testosterone in young females caused a decrease in phosphopyruvic acid formation. Cortisone and ACTH decreased the rate of this enzyme reaction in both sexes.

The authors are indebted to Armour and Co. for the generous gift of a sample of ACTH, and gratefully appreciate the crystalline cortisone donated by Merck and Co.

Received November 13, 1950. P.S.E.B.M., 1950, v75.

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Identification of Sterols on Filter Paper and Their Separation by Paper Partition Chromatography.* (18347)

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Partition paper chromatography, in which the partition is between the water of the hydrated cellulose of the filter paper and the organic mobile solvent, has been applied to a wide variety of chemical compounds(1,2). However, the separation and identification of steroids by this method is difficult, due to extremely low solubilities in H_2O and to a lack of good color reactions capable of detecting these compounds on filter paper. Recently, paper partition chromatography has been successfully applied to *keto steroids* by the formation of hydrazones with Gerard's Reagent T prior to chromatography(3) and by the use of non-aqueous solvent systems (4). This communication describes methods and conditions for the identification and separation of several non-keto steroids on a micro scale on filter paper.

Identification. Two satisfactory methods were developed for the identification of cholesterol, ergosterol, 7-dehydrocholesterol, vit. D_2 and vit. D_3—using Whatman's No. 1

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* The work reported in this paper was supported by a grant from the Central Scientific Fund of the State University of Iowa College of Medicine.

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filter paper. In the first, the paper containing the compound spots was suspended 5 cm above a bromine solution (0.25% Br_2 in pyridinesulfate dibromide, or 2% Br_2 in $CHCl_3$ or CCl_4, at 40°C) for 30 seconds. The paper was next sprayed with a saturated solution of KI in methyl alcohol and then with a 20% starch solution. The compound spots become dark blue while the surrounding area remains colorless or sometimes becomes light blue. The color fades with time and drying to a reddish brown. Ten gamma cholesterol spots can be detected; the method is even more sensitive for ergosterol. A more sensitive method involves spraying the sterol spots with a 20% solution of antimony pentachloride in chloroform. This color reaction was discovered and utilized by Steinle and Kahlenberg for the colorimetric determination of cholesterol(5). Immediately after spraying with the $SbCl_5$ solution, cholesterol develops a brown color, which darkens somewhat on standing, while ergosterol and 7-dehydrocholesterol give a purplish-red color which changes to a dark blue on standing; vit. D_2 and D_3 develop a pink color which changes to brown with standing. With 5 microliter spots, cholesterol can be detected in 5 γ concentrations, vit. D_3 in 3 γ, vit. D_2 in 2 γ, 7-dehydrocholesterol in 1 γ, and ergosterol in concentrations as low as 0.5 γ.

Separation. In attempting to determine a method for the separation of the above sterols, ascending(6), descending(7), and circular chromatograms(8) were run for any one solvent combination. Over 100 solvent combinations of the following solvents with one another were investigated: phenol, methanol, ethanol, n-propanol, n-butanol, t-amyl alcohol, benzene, xylene, toluene, acetone, carbon tetrachloride, cyclohexanone, chloroform, ether, petroleum ether (Skellysolve B), p-cresol, o-nitrotoluene and pyridine. The chief difficulty encountered was that most of the solvent combinations tried gave either no migration of the sterols or migration to the solvent front with no separation. The difficulty in separating the sterols is, at least in part, due to the great similarity of the 5 compounds studied. The only group here which tends to affect the R_f value is the hydroxyl at carbon atom 3, and this group is common to all the sterols studied. Furthermore, hydroxyl groups have a comparatively small effect in comparison to other substituent groups; alkane groups exert a very small effect in altering the R_f value(3). Water seems to have a striking effect on the migration, in that solvent combinations containing large proportions of water gave no migration, and as the water/organic ratio of the solvent combination grew smaller, migration occurred.

Two solvent combinations tried gave definitive separations; one was: phenol, 13.5 parts by volume; methanol, 30; water, 56.5. It was necessary to measure these components quantitatively, for varying the parts of phenol by one gave markedly different results. Using ascending chromatograms, this combination separated 7-dehydrocholesterol from cholesterol and ergosterol, using 10 μg quantities of each. The 7-dehydrocholesterol advanced slightly behind the solvent front, while the other 2 did not migrate at all. With phenol 14.0, methanol 30, and water 56 parts, only ergosterol remained behind, while 7-dehydrocholesterol and cholesterol were slightly behind the solvent front.

Using a solution of 80 parts methanol, 10 parts benzene and 10 parts petroleum ether (acid washed Skellysolve B, b.p. 60-70), 10 μg each of vit. D_3, cholesterol and 7-dehydrocholesterol were separated and distributed in 3 bands—from the solvent front on backward—on a circular chromatogram. The circular filter paper (12.5 cm) was placed over an evaporating dish (containing the solvent) which in turn was placed in a similar evaporating dish containing ice water. A 5-liter beaker was inverted over the whole set-up (Fig. 1). Antimony pentachloride in $CHCl_3$ was used to identify the various bands. Cholesterol could be separated from ergosterol

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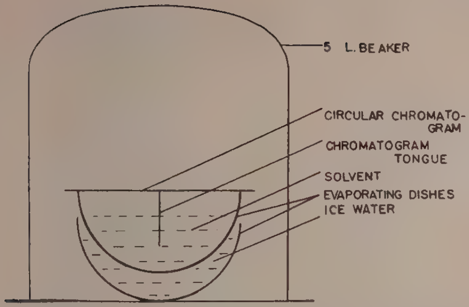

Fig. 1.
Apparatus for chromatographic separation of steroids.

using the same solvent and conditions. The importance of water-vapor and/or temperature was demonstrated, since no separation of cholesterol, ergosterol and vit. D_3 could be obtained on a circular chromatogram at 2°C in a cold room, nor at room temperature (approx. 30°C with the icebath eliminated).

Recently, Kostir and Slavik(9) devised a method for paper chromatography in which the partition is between 2 organic phases—one stable (acetylated filter paper), and the other mobile—in distinction to the ordinary partition between the water of the hydrated cellulose of the filter paper and the organic mobile solvent. They state that this method should enable one to separate compounds having low water solubility such as the fatty acids, steroids, etc., which are not resolved in the water-organic partition. We could not effect separation of sterols using this method. We think it advisable, however, to investigate this aspect of chromatographic separation of sterols further.

Summary. Methods for identification and separation of several non-keto steroids in 10 μg quantities on paper chromatograms are described.

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Received November 13, 1950. P.S.E.B.M., 1950, v75.

Effect of Cortisone Acetate upon Plasma Amino Acids in the Eviscerate Rat. (18348)

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The level of blood amino acids in the eviscerate-adrenalectomized rat is lower than that of non-adrenalectomized, eviscerate rats (1,2). The administration of adrenal cortex extract to eviscerate and to adrenalectomized-eviscerate rats causes a rise in plasma amino acids(2). In the present study, it was shown that the administration of cortisone acetate to eviscerate and to adrenalectomized-eviscerate rats caused a rise in plasma amino acids.

Methods. Male rats of the Sprague-Dawley strain were eviscerated at 250 g weight. Adrenalectomy was performed at the same operation. Immediately following operation, all of the animals were given continuous intravenous infusions of 0.9% sodium chloride with glucose and insulin. The volume was 20 cc per 24 hours per rat. The glucose load was 40 mg per 100 g of rat per hour, and the insulin dose (Regular Insulin, Lilly) was 4 units per 24 hours per rat. The details of the methods have been described(3,4). Cortisone acetate (Merck) in saline suspension was administered by subcutaneous injection at the beginning of the experiment and 6 hours later and 24 and 30 hours later in those animals which were studied for 48 hours. Eviscerate rats with and without cortisone acetate were studied simultaneously. Samples of blood

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TABLE I. Effect of Cortisone Acetate Upon Plasma Amino Acids Following Evisceration.

Group	Operation	No. rats	Hr	Cortisone mg/24 hr	Amino N mg % avg and range
1	Eviscerate	22	24	0	8.03 ± .24
2		11	24	5	8.31 ± .29
3		22	24	10	9.57 ± .30
4		12	48	0	13.15 ± .59
5		13	48	10	16.63 ± .39
6	Eviscerate-adrenalectomized	21	24	0	5.80 ± .20
7		11	24	5	7.71 ± .26
8		12	24	10	9.47 ± .45

were taken from the abdominal aorta at the end of each experiment. Plasma amino nitrogen was determined by the method of Hamilton and Van Slyke(5).

Experiments and results. The data are summarized in Table I.

Group 1 consisted of 22 eviscerate rats without cortisone which were observed for 24 hours. In Group 2, 11 eviscerate rats treated with 5 mg of cortisone acetate during 24 hours showed a statistically insignificant rise in plasma amino nitrogen. In Group 3, the treatment of 22 eviscerate rats with 10 mg of cortisone acetate during 24 hours caused a significant rise in plasma amino nitrogen above the average values of Group 1.

Group 4 consisted of 12 eviscerate rats without cortisone which were observed for 48 hours. The values for plasma amino nitrogen were significantly higher than at 24 hours. In Group 5, 13 eviscerate rats treated with 10 mg of cortisone acetate daily for 48 hours showed significantly higher values for plasma amino nitrogen than did the control animals of Group 4.

Group 6 consisted of 21 adrenalectomized-eviscerate rats without cortisone which were observed for 24 hours. The average value for plasma amino nitrogen was significantly lower than the average value for the non-adrenalectomized rats of Group 1. In Group 7, the treatment of 11 adrenalectomized-eviscerate rats with 5 mg of cortisone acetate each during 24 hours caused a significant rise in plasma amino nitrogen above the untreated controls (Group 6) and almost to the average values for the untreated non-adrenalectomized rats of Group 1. In Group 8, the treatment of 12 adrenalectomized-eviscerate rats with 10 mg of cortisone acetate each during 24 hours caused an average rise in plasma amino nitrogen which was significantly above the values for the untreated controls (Group 6) and significantly above the average values for the untreated non-adrenalectomized controls of Group 1.

Discussion. In support of earlier observations(1,2), these results show that the level of blood amino acids in the eviscerate rat is suppressed by adrenalectomy. It was further shown that the administration of cortisone acetate caused a rise in the concentration of plasma amino acids. It seems probable that these changes in the concentration of plasma amino acids represent an effect of the adrenal cortical hormones upon the equilibrium between free amino acids and proteins. Since the liver and other intraabdominal organs had been removed, it is clear that the effect of the cortical hormones upon nitrogen metabolism is not limited to these tissues. Long, Katzin and Fry(6) recognized the possibility that the cortical hormones may stimulate the catabolism of proteins in the peripheral tissues. It is also possible that the cortical hormones inhibit the rate of synthesis of proteins from amino acids(7) and thereby cause an increase in the concentration of free amino acids in the body water.

The possibility should be recognized that the observed changes might have been due to shifts in the amount of diluent (water) of the blood amino acids. The intake of water was

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constant in all of the animals. No differences in the excretion of water as related to the presence and absence of the adrenal glands and to cortisone were observed. The amounts of amino nitrogen in the urine were negligible. It seems probable that the amino acids of the plasma would move freely with water lost into the extracellular fluid of the tissues, but this is uncertain. The average increase in concentration of plasma amino acids caused by treating adrenalectomized-eviscerate rats with cortisone was over 60 per cent. If this change were due to a relative loss of diluent, there should have been a significant change in the hematocrit and in the concentration of hemoglobin. Such was not observed. The factors responsible for the effects of adrenal cortical hormones upon the concentration of plasma amino acids remain to be fully elucidated, but we suggest that it probably represents an extra-hepatic effect of the hormones upon protein metabolism.

Summary. Male rats were eviscerated and eviscerated-adrenalectomized. All of the animals received continuous intravenous infusions of saline with glucose and insulin for 24 and 48 hours. The administration of cortisone acetate in doses of 5 and 10 mg per 24 hours to one rat of each pair caused significant increases in the concentration of plasma amino acids above the values for untreated animals. It is postulated that the observed change represents an extra-hepatic effect of cortisone upon protein metabolism.

Received November 14, 1950. P.S.E.B.M., 1950. v75.

Marked Acute Hyperglycemic Response of Depancreatized Chicks to Adrenal Cortical Extract (ACE).* (18349)

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Neither pancreatectomy nor alloxanization effects a persistent hyperglycemia in chicks. Birds chronically surviving these procedures exhibit normal blood glucose levels and glucose tolerance curves(1-6). The reasons for this remain obscure. Previously, Golden and Long demonstrated that adrenal cortical extract (ACE) elicits gluconeogenesis in normal chicks; moderate hyperglycemia supervenes (2). These workers did not study the response of pancreatectomized birds to ACE.

The possibility presents itself that ACE-induced hyperglycemia may be enhanced in depancreatized birds due to a relative insulin lack present despite normal control plasma glucose levels(7). Since such a finding would throw light on the metabolic relationships prevailing in pancreatectomized birds, we undertook to study their glycemic responses to ACE.

Methods. A total of 9 depancreatized and 8 unoperated (control) cockerels were used.

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* The department is supported in part by the Michael Reese Research Foundation.

† Aided by grants from the Life Insurance Medical Research Fund, National Heart Institute (H 626) and the Chicago Heart Association.

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TABLE I. Four Hour Hyperglycemic Responses of Depancreatized and Control Chicks to a Single 5 cc Injection of ACE.

Control birds				Pancreatectomized birds			
	Plasma glucose	Plasma glucose	ACE-induced		Plasma glucose	Plasma glucose	ACE-induced
Chick No.	Pre-ACE mg%	Post-ACE* mg%	Glucose Δ+ mg%	Chick No.	Pre-ACE mg%	Post-ACE* mg%	Glucose Δ+ mg%
1	218	250	+32	6	252	256	+4
2	250	250	0	7	235	332	97
3	243	272	29	8	237	330	93
4	178	260	82	9	202	342	140
5	152	230	78	10	203	273	70
1	200	238	38	11	200	301	101
2	239	277	38	6	286	346	60
3	224	262	38	7	190	251	61
				8	228	343	115
Mean	213	255	+41.9 ± 9.4†	Mean	226	308	+82.3 ± 13.1†
Range	152-250	230-277	0-82	Range	190-286	251-236	4-140

* 4 hr response.

+Δ is rise in plasma glucose conc.

† 9.4 and 13.1 are standard errors(9).

Pancreatectomy was performed via an abdominal incision under sodium pentobarbital anesthesia. All experiments were done at least 10 days after pancreatectomy, some several months thereafter. All birds exhibited normal feed intake, growth and development and exhibited no overt signs of abnormal metabolism. All birds in paired experiments were approximately the same age and weight. Completeness of pancreatectomy was confirmed postmortem; in 2 of the birds, small pancreatic remnants were found. Two series of experiments were done. In the first, a single 5 cc injection of adrenal cortical extract‡ was given and the 4 hour plasma glucose response analyzed. In the second, hourly ACE injections (1 cc) were given for 10 hours. Blood for plasma glucose analysis was drawn from an alar vein 1, 20 and 40 hours after the last ACE injection. Glucose was determined by the Somogyi-Shaffer-Hartman method§(8).

Results. The findings in the first series of experiments are summarized in Table I. In accord with other workers(2), we found that ACE induces a hyperglycemic response in normal chicks. Further in accord with previous results(1-6), the depancreatized chicks exhibited pre-ACE control plasma glucose levels within the normal range. Depancreatized chicks responded to a single 5 cc injection of ACE with a 4 hour rise in plasma glucose levels that was significantly greater than in the control cockerels (Table I).

This phenomenon was demonstrated far more clearly by the experimental procedure utilized in the second series (Table II). Thus 1 hour after the last of 10 hourly 1 cc injections of ACE, the depancreatized chicks showed a rise in mean plasma glucose from 234 to 617 mg% (controls: 223 to 403 mg). One operated bird exhibited a plasma glucose of 745 mg% at this time, an increment of 502 mg%. The rise in plasma glucose persisted in the pancreatectomized birds, as revealed by 20 and 40 hour analyses, whereas it was transitory in the controls (Table II). These differences are statistically significant(9).

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‡ We are indebted to Dr. Harold Hailman of The Upjohn Co., Kalamazoo, Mich., for liberal supplies of Lipo-Adrenal Cortex.

§ We wish to thank B. Huddlestun of the Department of Metabolic and Endocrine Research and M. Murakami of the Department of Biochemistry who did the sugar analyses in this study.

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TABLE II. Hyperglycemic Responses of Depancreatized and Control Chicks to 10 Hourly Injections of ACE (1 cc).

Pancreatectomized birds

Chick No.	Plasma glucose pre-ACE, mg%	Plasma glucose 1 hr post-ACE,* mg%	1 hr Δ mg%	Plasma glucose 20 hr post-ACE, mg%	20 hr Δ mg%	Plasma glucose 40 hr post-ACE, mg%	40 hr Δ mg%
12	212	630	+418	450	+238	260	+ 48
13	248	475	227	440	192	243	— 5
14	243	745	502	700	457	495	+252
Mean	234	617	+382	530	+296	333	+ 98
Range	212-248	475-745	227-502	440-700	192-457	243-495	—5 - +252

Control birds

Chick No.	Plasma glucose pre-ACE, mg%	Plasma glucose 1 hr post-ACE,* mg%	1 hr Δ mg%	Plasma glucose 20 hr post-ACE, mg%	20 hr Δ mg%	Plasma glucose 40 hr post-ACE, mg%	40 hr Δ mg%
15	217	390	+173	228	+11	205	—12
16	207	405	198	228	21	205	— 2
17	245	415	170	298	58	185	—60
Mean	223	403	+180	251	+30	198	—25
Range	207-245	390-415	170-198	228-298	11-58	185-205	—2 - —60

* After last of 10 consecutive hourly inj.

Discussion. Injection of ACE into depancreatized chicks results acutely in a hyperglycemia significantly greater in degree and longer in duration than that exhibited by unoperated control birds. This response corresponds to that observed in partially depancreatized rats given ACE(7). The plasma glucose levels in our operated, ACE-treated chicks are in the diabetic range. These findings supervene although the depancreatized chicks have normal pre-ACE plasma glucose levels and glucose tolerance curves. They indicate a relative insulin lack and a latent diabetic tendency (subdiabetes), in depancreatectomized birds(4,7,10-14).

Summary. Injection of ACE into depancreatized cockerels induces a marked acute hyperglycemic effect, significantly greater in degree and longer in duration than in unoperated control birds.

We thank other members of the department's atherosclerosis research team for their cooperation in this study, including Miss C. Bolene, Deborah V. Dauber Research Assistant, and Messrs. P. Johnson and G. Crowley, laboratory technicians.

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Received November 14, 1950. P.S.E.B.M., 1950, v75.

Hemorrhagic Diathesis with Ascorbic Acid Deficiency During Administration of Anterior Pituitary Corticotropic Hormone (ACTH).* (18350)

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The effect of the administration of anterior pituitary corticotrophic hormone (ACTH) on the metabolism of ascorbic acid is, thus far, a highly controversial subject. Most of the reports available are based on short term experiments in animals. Sayers *et al.*(1) have shown that the injection of a single dose of ACTH in rats and guinea pigs is followed by a prompt fall in the ascorbic acid content of the adrenal cortex, while other organs are not equally affected. Booker *et al.*(2) have observed decreased ascorbic acid content of the adrenals, low plasma level and initially high urinary excretion of ascorbic acid in animals given day-to-day injections of ACTH. Correlated with these findings is the observation by Fortier and Skelton(3) that the injection of adrenal cortical extract raises the plasma level of ascorbic acid, even after adrenalectomy, thus suggesting that ascorbic acid may be mobilized from the tissues in any "hyperadrenal state." Reports on the effect of ACTH on the metabolism of ascorbic acid in man are contradictory. Bodansky(4) reports no significant changes in urinary ascorbic acid excretion in 2 patients treated with ACTH, but the additional administration of cortisone in one of them induced increased excretion of the vitamin. In contrast to these findings, Beck *et al.*(5) and Johnson(6) have stated that a prompt sustained increase in urinary ascorbic acid excretion was noted in 17 out of 23 patients given ACTH. Cortisone produced no such effect. The question is of importance because, if ACTH may determine increased and sustained excretion of ascorbic acid in the urine, depletion of the vitamin might possibly occur. For this reason we consider it of value to present two patients who, while on ACTH therapy, developed a hemorrhagic syndrome accompanied by evidence of ascorbic acid depletion.

The first case, M.C., a 58-year-old male, received approximately 2.7 g of ACTH (Wilson) over a period of 125 days for the treatment of lymphosarcoma with secondary hemolytic process. The Wilson preparation utilized in this case is stated(7) to possess twice the potency of the Armour standard LA-1-A on a weight for weight basis. Small ecchymoses were first noticed over the pretibial region on the 115th day of therapy. Laboratory examination revealed a moderate degree of liver damage (cephalin-cholesterol flocculation test: 4+; thymol turbidity: 8 units; B.S.P.: 15% retention after 45 minutes following the injection of 5 mg/kilo weight of the dye). Studies of the hemostatic mechanisms (Table I) indicated delayed clotting time in silicone-coated test tubes, decreased plasma prothrombin and labile factor activity

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* Supported by grants from the Charlton Fund and the American Cancer Society, Massachusetts Division.

† This work was completed during the tenure of a Damon Runyon Senior Clinical Research Fellowship, administered by the American Cancer Society.

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TABLE I. The Status of the Various Factors of Hemostasis in the 2 Patients Presenting Hemorrhagic Manifestations in the Course of Therapy with Anterior Pituitary Corticotropic Hormone (ACTH).

	Patient M.C. age 58	J.P.S. 54
Platelets, per cmm (×1000)	379 to 438	398.5
Bleeding time, right ear, min.	4½	3½
left " "	3½	2½
Tourniquet test(1), petechiae	5	12
Coagulation time glass test tube, min.	8	9
Silicone-coated test tube, min.	56	34
Clot retraction(2), %	51	47
Prothrombin activity of plasma, %	32	69
Prothrombin activity of serum, %	7	13
Serum/plasma prothrombin activity ratio, ×100	21.7	18.8
Plasma labile factor activity, %	56	75
Serum " " " "	14	8
Serum/plasma labile factor activity ratio, ×100	25	10.7
Accelerator effect of serum, %	45	53
Antithrombin activity, plasma	0	0
Antithromboplastin activity, plasma	0	0
Fibrinogen, mg %	483.0	278.0
Fibrinolytic activity, plasma %	22	13

1. No. of petechiae in a circular area of 1″ diameter, after 10 min. application of supradiastolic pressure.

2. Volume of serum expressed from 1 ml of blood at end of one hr after completion of coagulation, multiplied by 100.

A brief description of the technics used for these determinations and their bibliographic source have been presented in a previous paper(10).

and moderately increased plasma fibrinolytic activity. While this picture was consistent with that found in moderate liver dysfunction(8,9), it did not seem to explain the occurrence of spontaneous hemorrhagic manifestations observed in the patient, because the capillary resistance appeared normal and the depletion of the various coagulation factors was above the recognized critical level(10). Moreover, while the tourniquet test produced only a few scattered petechiae over the forearm, direct trauma to the skin, exemplified by a light prick not strong enough to cause bleeding produced, over a period of hours, a large extravasation of blood surrounding the traumatized zone. Notwithstanding a normal intake of ascorbic acid in the diet (approximately 85 mg a day), this finding was very suggestive of vascular injury as found in scurvy. Plasma ascorbic acid level, determined by the colorimetric method of Mindlin and Butler(11) was 0.33 mg %. Determinations on three successive days gave comparable figures of 0.17 mg %, 0.28 mg % and 0.19 mg %. The urinary elimination of ascorbic acid, determined with the titrimetric method of Harris and Ray(12), was 0.99 mg per 24 hours. The level of ascorbic acid was then determined in the plasma and urine of the patient for the five hours following the intravenous injection of 1 g of ascorbic acid (Table IIa). The total elimination of ascorbic acid in the urine for the 5-hour period was 69.8 mg; the total 24-hour excretion was 102.6 mg. The same procedure was repeated 2 days later, when the tissues of the patient had been presumably saturated with ascorbic acid. The results obtained are indicated in Table IIb. A total amount of 272.8 mg was passed in the urine within 5 hours and 358.0 mg within 24 hours following the injection.[§] The various factors of hemostasis studied were not significantly modified following the administration of ascorbic acid, but no further spontaneous hemorrhagic manifestations occurred. Nor was it any longer possible to induce subcutaneous hemorrhages with minor trauma of the skin (pin pricks).

The second case, J.P.S., a 59-year-old male, was a patient with chronic rheumatoid arthritis of 20 years duration displaying both marked joint deformity and progressive disability. He had been treated with pregnenelone acetate for 5 weeks prior to admission. He was given over a period of 9 days a

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[§] For purposes of comparison a similar test was made in a healthy subject, 33 years old. The results are given in Table IId. 367.7 mg of ascorbic acid were eliminated within 5 hrs and 561.3 mg in the 24 hrs following intrav. injection of 1 g of ascorbic acid.

TABLE II. Urine and Plasma Ascorbic Acid Levels at Rest and Following the Intravenous Administration of 1 g of Ascorbic Acid in the Patients M.C. and J.P.S., and in a Healthy Subject.*

Time (hr)	Before	After	1	2	3	4	5
(a) Patient M.C.							
plasma, mg %	0.17	29.0	5.4	2.2	0.9	0.7	0.25
urine, " "	0.11†						14.8
(b) Patient M.C. (2 days after first test)							
plasma, mg %	0.33	30.2	9.3	5.3	2.0	1.6	0.8
urine, " "	0.29†						52.8
(c) Patient J.P.S.							
plasma, mg %	0.4						
urine, " "	0.19†						11.3
(d) Healthy subject							
plasma, mg %	1.2	32.7	11.7	7.6	5.9	3.5	2.1
urine, " "	2.86†						88.7

* Blood was collected before, immediately after, and at regular intervals of time following the intravenous injection of 1 g of ascorbic acid. The urine was voided before the injection and again 5 hours later.

† 24-hr collection.

total of 28 mg of a special ACTH preparation obtained from Dr. E. B. Astwood's Laboratory and stated to be 25 to 30 times more potent than the Armour standard La-1-A on a weight for weight basis. On the ninth day of therapy, petechiae and small ecchymoses appeared over both legs and light pricking of the skin caused, as in the first patient, delayed appearance of large petechiae. The results of detailed investigations of the hemostatic mechanism are shown in Table I, the only important finding being subnormal capillary resistance to stress. The patient had an ascorbic acid intake of approximately 75 mg a day. Ascorbic acid determinations were run in plasma and urine and the results are presented in Table IIc. A tolerance test of ascorbic acid was refused by the patient who left the Hospital shortly afterwards. Following the administration of ascorbic acid, light pin pricking of the skin failed to cause appearance of petechiae.

Discussion. The diagnosis of ascorbic acid deficiency is a difficult one, mainly because of the limited reliability of the laboratory methods employed for its determination. In these 2 patients, however, we feel that this diagnosis was justified by a series of findings: (1) the occurrence of hemorrhagic manifestations in patients with only moderate abnormalities of other factors of hemostasis, especially following minimal trauma of the skin; (2) the low plasma ascorbic acid level and the low urinary excretion of the vitamin in these 2 patients; (3) the low plasma ascorbic acid tolerance curve (in the one case studied) and limited urinary excretion of ascorbic acid following the administration of 1 g of the vitamin intravenously; (4) the regression of the typical hemorrhagic manifestations induced by trivial trauma of the skin after the administration of ascorbic acid.

It must be emphasized that ascorbic acid deficiency is not to be considered a common occurrence during administration of ACTH. In at least 3 more patients treated with high doses of ACTH over a period of months we have not been able to detect any abnormality in the metabolism of ascorbic acid. This doubtless explains why this possible complication of ACTH therapy has been unrecognized thus far, when predisposing conditions are not present.

Summary. 1. Two patients developed hemorrhagic manifestations presumably due to ascorbic acid deficiency while receiving high doses of anterior corticotrophic hormone (ACTH). The criteria for such a diagnosis are discussed. 2. Ascorbic acid deficiency is probably not a common occurrence in the course of ACTH therapy, but should be considered if hemorrhagic manifestations appear during prolonged administration of the hormone, when other factors of hemostasis are above critical levels.

Received November 14, 1950. P.S.E.B.M., 1950, v75.

Serum Hepatitis in U. S. Troops in Germany.* (18351)

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Acute hepatitis continues to have a high prevalence among U. S. troops stationed in Germany(1). The institution of effective control measures has been made difficult by an inability to determine by clinical observations or by laboratory tests, the extent to which such cases represent naturally occurring infectious hepatitis (IH), or homologous serum jaundice (SH) or both. The purpose of this paper is to call attention to the proved existence of serum hepatitis in at least one instance among such personnel, as established by experiments in human volunteers.

The experiments involving the use of volunteers reported in this paper represent a continuation of similar studies on viral hepatitis begun in 1944 by Havens(2-5). The current study was initiated in the summer of 1949 with a view to obtaining a sample of icterogenic serum of the short incubation (IH) type for use in attempts to propagate this agent in developing chick embryos or in tissue culture. It was a surprise, therefore, when the positive sample obtained from a U.S. soldier in Germany, turned out to be the long incubation type. Because of its bearing on the control of this disease in our troops, this finding is reported.

Materials and Methods. Inoculum: Plasma samples were obtained from 2 soldiers (W.M. and J.W.), who contracted acute hepatitis in Germany†. There had been no reason to suggest clinically that their illness was serum hepatitis. The blood had been drawn on the fourth and second day of their illnesses respectively, as shown in Table I. The plasma samples had been frozen in a CO_2 dry ice box for 42 days and had been transported to this country in the frozen state, following which they were thawed, heated at 56° for 30 minutes, and tested for bacteriologic sterility.

Subjects. Volunteers, aged 21 to 28 years, were males in good physical condition and without histories of prior hepatitis. They were isolated for a period of 40 days following inoculation and observed clinically each week for a period of 10-15 weeks. *Method of inoculation*: Each sample was administered as follows: 0.5 ml subcutaneously, 0.1 ml intradermally, and 0.5 orally in 30 ml of skim milk. *Laboratory work*: Multiple tests of liver function were carried out at weekly intervals, or more often, for a period of at least 3 months.

Results. Three volunteers were inoculated with sample W.M., and 3 other volunteers with sample J.W. The results are shown in Table II. With sample W.M., none of the volunteers developed appreciable evidence of hepatitis. Minor symptoms, however, were encountered about 3 weeks after inoculation. One volunteer (A.B.) experienced anorexia, nausea, "dark urine," and tenderness over the liver unaccompanied by altered liver function tests, including bromsulfalein excretion, and at the same time a second volunteer (R.H.) had diarrhea but no other symptoms.

With serum J. W., all 3 volunteers eventually developed definite clinical and laboratory

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* Representing work done under the auspices of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, U. S. Army, Washington, D.C.

Appreciation is expressed to Mr. Allen Shank, Warden, and Dr. Leon A. Witkin, Physician, Federal Correctional Institution, Danbury, Conn., for their cooperation in this study.

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† These specimens were received from the Hepatitis Research Team at the 98th General Hospital, Germany. Acknowledgement is made to Dr. J. W. Colbert and Dr. Dorothy M. Horstmann for this material.

TABLE I. Data on Patients Whose Blood Was Used in Transmission Experiments.

			Character of hepatitis			
			Onset			
Patient	Age	History of inj., 1949	1949	Type	Severity of illness	Inoculum obtained 1949
W.M.	23	Penicillin treatment, August Dental treatment, July	26 Sept.	Insidious	Mild	30 Sept.
J.W.	30	Sutures for cut on leg, August Dental extraction, August	28 Sept.	”	”	30 Sept.

TABLE II. Results of Transmission Experiments in Human Volunteers.

			Experimental hepatitis					
			Early findings		Late findings			
Inoculum from donor	Method of inoculation	Volunteer	Day after inoc.	Type	Jaundice	Day after inoc. when clinical jaundice appeared	Highest bilirubin mg/100 ml	Final result
W.M.	Oral and P	A.B.	17	Anorexia, “dark urine,” tender liver	None			0
		R.H.	17	Diarrhea	None			0
		G.F.		None	None			0
J.W.	Oral and P	F.T.		None	Yes	53	2.96	+
		B.S.	18	Anorexia, tender liver, slight fever	Yes	68	9.96	+
		C.H.	19	Malaise, tender liver, slight fever	Yes	87	7.97	+

P = Parenteral inoculation by intradermal and subcut. inj.

evidences of jaundice with incubation periods of 53, 68, and 87 days, respectively. Two of these subjects (B.S. and C.H.) had also experienced early symptoms occurring about 3 weeks after inoculation and consisting of malaise, slight fever, back or epigastric discomfort, and tenderness over the liver without enlargement; one subject complained of early anorexia and nausea. In one of these volunteers (C.H.), similar symptoms recurred on the 73rd day after inoculation and persisted through the occurrence of definite jaundice on the 87th day. The preliminary symptoms in these 2 volunteers were not associated with alterations in liver function tests except for slight increases in thymol turbidity units in the serum of one. Elevation of values for thymol turbidity and 1-2 + cephalin cholesterol flocculation tests, however, preceded by several weeks the onset of clinical jaundice in both these volunteers.

A second experiment, details of which will not be given here, was later carried out in the hope of obtaining plasma containing the agent of I.H. In an attempt to avoid the production of serum hepatitis in these volunteers, the inoculum was given by the *oral* route alone, since I.H. is readily transmitted in this way while S.H. has been induced experimentally *only* by parenteral inoculation (6) with 2 possible exceptions(7,8). Three samples of plasma from 3 soldiers in the early stages of acute viral hepatitis were tested in 3 volunteers each by the oral route alone. None of these 9 volunteers developed clinical jaun-

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dice although transient symptoms and minor deviations in liver function tests did occur in 2 out of 3 volunteers in each group from 41 to 64 days after oral administration of the plasma and in one volunteer a serum bilirubin of 2.16 mg/100 ml was observed on the 44th day. The failure to demonstrate the agent of I.H. in these 3 acute phase blood samples can not be accepted as unequivocal evidence that it was not present since the number of volunteers employed was small. The results obtained lend some weight to those of experiment 1, *i.e.*, that serum hepatitis is at least in part responsible for jaundice in U.S. troops in post-war Germany.

Discussion. Our knowledge of the viruses of I.H. and S.H. is extremely limited and to date has been accumulated almost entirely from work with volunteers. The previous experiments have been well reviewed in a recent report by Havens(6).

The results of the present experiment establish the existence of serum hepatitis, or at least "long incubation period" hepatitis, in U.S. troops stationed in Germany in 1949 where viral hepatitis has been endemic and highly prevalent among U.S. soldiers(1). There is no evidence presented by this or other published studies which indicates the extent to which serum hepatitis may be present among these troops although there is considerable circumstantial evidence from other sources which would suggest that serum hepatitis is common(1).

The soldier whose plasma contained an icterogenic agent of the long incubation period type had been exposed in the 2 months preceding his jaundice to dental instruments for a tooth extraction, as well as to suture needles, either of which may have been contaminated with icterogenic blood. Possible transmission of this disease by dental equipment as well as by surgical instruments should thus be borne in mind.

The occurrence of clinical symptoms and of tenderness over the liver was noted 17-20 days after inoculation in 2 of 3 volunteers who developed definite jaundice at a much later date and similar findings were also noted in 2 of 3 volunteers who never developed definite jaundice. These early changes in the course of serum hepatitis have previously been noted in volunteers studied by Neefe *et al.*(9) and it is known that the causative agent is present in the blood of such volunteers during the first months after inoculation, as demonstrated by Paul, *et al.* and others(6).

Summary. The presence of serum hepatitis (SH), or at least of long incubation period hepatitis, among the hundreds of cases of acute hepatitis which have been occurring annually in U.S. troops stationed in Germany has been established by human volunteer experiments using as the inoculum acute phase plasma obtained from a soldier who had contracted hepatitis in that area. In one experiment, one of 2 sera so tested produced clinical jaundice in 3 of 3 volunteers following incubation periods of 53, 68 and 87 days, respectively. Symptoms suggesting mild hepatitis occurred about 3 weeks after inoculation in 2 of these same 3 volunteers.

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Received November 15, 1950. P.S.E.B.M., 1950, v75.

Retardation of Tumor Growth in Mice by Oral Administration of Methyl Androstenediol and Methyl Testosterone. (18352)

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The retardation of growth of transmissible tumors of mice by steroid hormones has been described by several investigators(1-4), and favorable results in the treatment of metastatic breast cancer in women by the parenteral injection of methyl androstenediol have been reported by Homburger, Kasdon and Fishman(5). The present report presents ex-

TABLE I. Retardation of Tumor Growth in Mice by Methyl Androstenediol and Other Compounds.

Tumor	Measurement after, days growth	Compound	No. of animals	Sex	Dosage	Avg tumor size (cu mm)	Range
Sarcoma 180	13	Methyl androstenediol	20	♂	.06*	603	(413-817)
		'' ''	5	♂	.03*	618	(507-778)
		'' ''	5	♀	.03*	629	(569-728)
		'' ''	6	♂	.015*	1530	(1200-2200)
		'' ''	5	♀	.015*	1517	(1065-2196)
		'' ''	12	♂	100†	589	(373-687)
		Methyl testosterone	6	♂	100†	645	(406-702)
		Urethane	6	♂	.15*	746	(504-1144)
		Controls	25	♂	—	2130	(1508-3558)
		''	5	♀	—	1635	(1490-2980)
Mammary gland adenocarcinoma C3H-BA		Methyl androstenediol	4	♀	.06*	205	(120-390)
	11	Controls	4	♀	—	1560	(1170-2400)
	15	Methyl androstenediol	6	♀	.06*	576	(430-730)
		'' ''	6	♀	100†	333	(120-420)
		Controls	6	♀	—	1383	(1050-1872)
	18	Methyl androstenediol	6	♂	.06*	505	(336-840)
		'' ''	6	♂	.03	1345	(847-1843)
		Controls	6	♂	—	2852	(2345-3183)
	15	Methyl testosterone	5	♂	100†	468	(162-686)
		Controls	5	♂	—	1295	(1081-1860)
Adenocarcinoma EO-771	11	Methyl androstenediol	5	♂	.06*	245	(125-330)
		Controls	5	♂	—	600	(400-1092)
Sarcoma 37	8	Methyl androstenediol	4	♂	.06*	375	(140-616)
		Controls	4	♂	—	699	(480-896)

* % in diet. † mg/kg/day orally.

periments in which the growth of transplantable mouse tumors, sarcoma 180 and adenocarcinoma C3H-BA, were retarded when methyl androstenediol (3B-17 B-17 methyl androstenediol ("Methostan") or methyl testosterone ("Oreton M") was given orally to mice bearing subcutaneous implantations of the tumors.

Sarcoma 180 and sarcoma 37 were implanted in albino mice, adenocarcinoma C3H-BA in C3H mice, and adenocarcinoma EO-771 in C57 black mice.* Minimal implantations were made subcutaneously with an 18 gauge trocar in the right axillary region of young adult (18-20 gram) mice, and administration of the test substances was started 24 hours later. Tumor volume was calculated from measurements made in 3 diameters with vernier calipers. Methyl androstenediol or methyl testosterone was dissolved in warm corn oil and mixed with the pulverized stock diet (Wayne Fox Food) and fed *ad libitum* or administered daily orally in 0.5 ml of corn oil by stomach tube and syringe. Urethane, used as a control, was dissolved in a small quantity of water, mixed with the diet, and fed *ad libitum*. It was estimated that each mouse ate about 3 grams of food a day. Thus the daily intake of the steroids by mice fed the 0.06% diet was approximately 100 mg/kg a day. No marked loss of weight of the animals was noted during the treatment. Results showing the tumor retarding effect of the compounds when given by the oral route are shown in Table I.

From the data in Table I, it is seen that methyl androstenediol, when given orally, has

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a distinct retarding effect on the growth of both sarcoma 180 and adenocarcinoma C3H-BA. This effect is apparently dependent upon the amount administered, little retardation being noted when the diets containing 0.015% was fed. Methyl testosterone, likewise, retarded the growth of these tumors when 100 mg/kg daily was given by the oral route. Growth of adenocarcinoma EO-771 and sarcoma 37 was not greatly retarded by methyl androstenediol given in concentrations which markedly impaired the growth of the other tumors.

The retarding effect of the compounds on the tumors is confined essentially to the first two weeks of treatment; after this time, growth proceeds at a rapid rate in spite of continued treatment. When implantations were allowed to grow for 5 days before oral treatment with methyl androstenediol was begun, little or no inhibition of growth was seen.

No retardation in growth of sarcoma 180 has occurred in repeated experiments in which either methyl androstenediol or methyl testosterone has been administered *subcutaneously* in 100 mg/kg daily doses for 13 days.

Summary. The growth of sarcoma 180 or adenocarcinoma C3H-BA is markedly retarded in mice by *oral* administration of methyl androstenediol or methyl testosterone in doses approximating 100 mg/kg daily, when treatment is started the day following implantation. Inhibition of sarcoma 37 and adenocarcinoma EO-771, under similar conditions, is slight and of short duration. No retardation of growth of sarcoma 180 was noted when 100 mg/kg doses of the compounds were administered daily by *subcutaneous* injection.

Received November 17, 1950. P.S.E.B.M., 1950, v75.

Use of Bromsulphalein for the Measurement of Proteolytic Activity.
(18353)

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Disodium phenoltetrabromophthalein disulfonate (Bromsulphalein), widely used as a test of hepatic function(1), has been found to be an enzyme inhibitor(2). In the study of this property, it was noted that protein could be precipitated completely from an acid solution by addition of this indicator, and that the color intensity of an alkaline solution of the precipitate was proportional to the amount of protein present. Carroll(3) by reporting recently the effect of pepsin on the binding of bovine albumin with the dye orange I, suggested the present investigation.

Experimental. 1. *Relation of protein concentration to amount of Bromsulphalein in precipitate.* Casein prepared according to Hammarsten(4) (Kahlbaum) was dissolved in aqueous 0.1% Na_2CO_3 solution so that 1 ml contained the amount of protein shown on the abscissa of Fig. 1. In 9.8 x 1.2 cm test tubes, 1 ml of casein solution and 1 ml of distilled water were mixed with 2 ml of indicator solution made by adding 3 ml of 5% aqueous Bromsulphalein (Hynson, Westcott, and Dunning) to 197 ml pH 2.5 citrate-phosphate buffer (McIlvaine)(5). The tubes were centrifuged at 2500 rpm for 10 minutes, inverted, and allowed to drain for several minutes, after which the inner walls of the tubes were wiped dry with absorbent cotton on an applicator stick. The precipitates at

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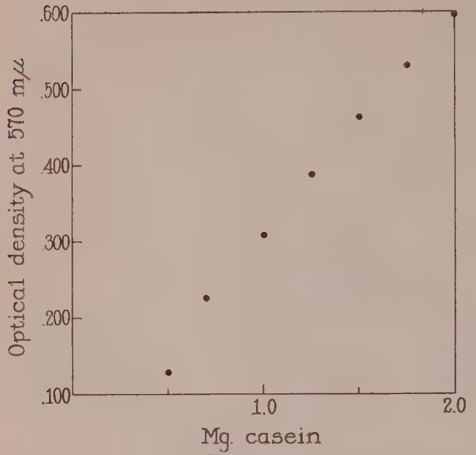

FIG. 1.

Relation of optical density of alkaline solution of precipitate to amount of casein.

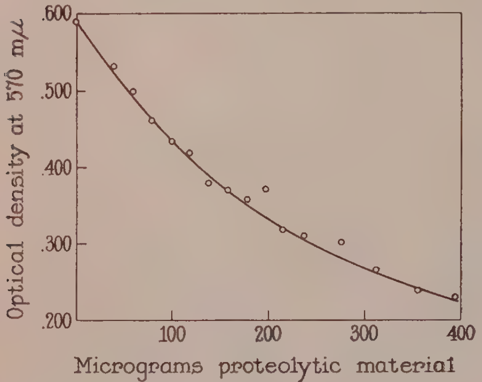

FIG. 2.

Relation of optical density of solution of precipitate from 2 mg of casein to amount of proteolytic material.

the bottoms of the tubes were dissolved in 2 ml, 10% NaOH, transferred quantitatively to a graduated cylinder, and diluted to 100 ml with distilled water. After thorough mixing, a portion of the solution was transferred to a 15 x 1.6 cm cuvette, and the optical density was measured at a wavelength of 570 mμ in the Coleman, Jr. Spectrophotometer (Model 6A). Fig. 1 shows the optical density obtained by following the above procedure with concentrations of casein between 0.50 and 2.0 mg.

2. *Measurement of proteolytic activity.* 50 mg of a commercially available proteolytic enzyme preparation (Trypsin "pure," Amend) were suspended in 100 ml of distilled water, and allowed to stand at room temperature (26-28° C) for 2 hours with occasional shaking. The resulting opalescent solution was filtered through washed cotton to remove particulate matter, and a portion evaporated, then dried at 120° C to obtain the dry weight. The filtrate was found to contain 394 μg per ml. Water dilutions of the remaining solution were made to contain in 1 ml, the weights indicated on the abscissa of Fig. 2. To 1 ml of each dilution was added 1 ml of solution containing 2 mg casein in 0.1% Na_2CO_3. Following incubation for 30 minutes at 37° C the reaction was stopped, and the remaining unchanged protein precipitated, by the addition of 2 ml of Bromsulphalein solution. The remainder of the procedure was carried out as in Section 1.

Fig. 2 shows a typical curve relating optical density of a solution of remaining unchanged protein to dry weight of proteolytic preparation. The amount of Bromsulphalein precipitated by the proteolytic preparation alone was in this instance negligible.

Comment. In addition to casein, all the protein preparations tested, including human serum albumin, crystalline bovine plasma albumin (Armour), ovalbumin, hemoglobin, bovine gamma globulin (Armour), normal human serum, and lysozyme also precipitate with Bromsulphalein from acid solution. In the case of gelatin, high speed centrifugation may be used to pack a finely divided precipitate. Curves similar to Fig. 2 can be obtained with solutions of crystalline pepsin, commercial papain, and ficin preparations, and the proteolytic reaction may be carried out at the optimum pH for the enzyme tested provided the precipitation is conducted at or near pH 2.5. It is also possible to detect as little as 10 μg of protein, measuring by difference, the amount of color removed from a known quantity of Bromsulphalein. It should be noted that the concentration of Bromsulphalein must be carefully adjusted in these experiments, as large excesses of this indicator may result in incomplete precipitation of protein.

Summary. From acid solutions, Bromsulphalein is precipitated in amounts proportional to the protein present. This reaction has been used to measure proteolytic activity, and could be adapted to the quantitative determination of minute amounts of protein.

Received November 17, 1950. P.S.E B.M., 1950, v75.

Effect of Eserine and Atropine on ACTH Release. (18354)

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Recent observations suggest that hypothalamic centers might take part in the control of the corticotrophic function of the hypophysis, through the release into the pituitary portal system of an unknown stimulating agent(1,2). Adrenalin and histamine have been so far investigated in this respect with negative results; adrenolytic(2-4) as well as antihistaminic(5) agents failing to prevent a stress-induced release of ACTH. The aim of the following experiments was to study the role of acetylcholine in the regulation of ACTH release. Using Sayers' test(6) as an index of corticotrophic function, we have measured, in the rat, the adrenal ascorbic acid response to the independent and simultaneous administration of a parasympathomimetic drug (eserine) and an anticholinergic agent (atropine).

One hundred twenty male piebald rats of an average weight of 130 g were subdivided into groups of 8 animals. Eserine salicylate was subcutaneously injected at a dose level of 0.03 mg/100 g, atropine sulphate at a dose of 20 mg/100 g. The animals, previously fasted for 24 hours, were killed by bleeding one hour after the injection and the adrenal ascorbic acid determined according to Roe and Kuether's method.

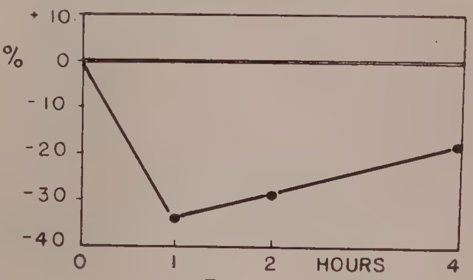

Fig. 1.
Effect of eserine salicylate (0.03 mg/100 g) on adrenal ascorbic acid concentration.

A maximal ascorbic acid depletion (Fig. 1) was observed one hour after the administration of eserine; it was followed in the next hours by a progressive return to normal level.

Atropine (Table I), far from preventing the eserine-induced depletion of adrenal ascorbic acid, increased it significantly and was by itself responsible of an intense discharge.

In order to eliminate the possibility of a direct adrenal ascorbic acid depleting effect of atropine, eventuality suggested by di Mattei's(8) and Frommel's(9) observations on the devitaminizing action of certain drugs, we studied in a final experiment the adrenal ascorbic acid response to atropine, 48 hours after hypophysectomy.

A complete inhibition (Table II) of the

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* Canada-UNESCO Research Fellow.

† This work was done during the tenure of an American Heart Association Research Fellowship.

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TABLE I. Effect of Atropine on the Adrenal Ascorbic Acid Response to Eserine.

Treatment	Adrenal ascorbic acid, γ/100 mg
None	468.6 ± 23.2
Eserine*	404.5 ± 29.8
Atropine†	318.4 ± 20.9
Eserine + atropine	281.9 ± 10.8

* Eserine (salicylate) 0.03 mg/100 g.
† Atropine (sulphate) 20 mg/100 g.

TABLE II. Effect of Hypophysectomy on the Adrenal Ascorbic Acid Response to Atropine.

Treatment	Adrenal ascorbic acid, γ/100 mg
None	488.4 ± 16.5
Atropine*	286.0 ± 13.8
Hypophysectomy	540.0 ± 15.5
Hypophysectomy + atropine	582.7 ± 17.7

* Atropine (sulphate), 20 mg/100 g.

usual response to atropine was observed under these conditions.

The pituitary-mediated adrenal ascorbic acid depleting effect of atropine as well as its failure to prevent the response to eserine, seem to preclude the possibility of a cholinergic control of ACTH release.

Summary. The adrenal ascorbic acid response to the independent and simultaneous administration of eserine and atropine was studied in the rat. (1) The eserine-induced adrenal ascorbic acid depletion is enhanced by simultaneous administration of atropine. (2) Atropine alone causes a significant fall of the adrenal ascorbic acid concentration. This fall is prevented by hypophysectomy. (3) Our results seem to preclude the possibility of a cholinergic control of ACTH release.

This investigation was supported by a research grant from the National Cancer Institute of Canada.

Received November 20, 1950. P.S.E.B.M., 1950, v75.

Comparative Utilization of Fructose and Glucose Given Intravenously.*
(18355)

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It has long been known that fructose, despite its slower rate of intestinal absorption (in the rat) is converted to hepatic glycogen more rapidly than glucose(1). *In vitro* experiments using rat liver slices and homogenates(2) have shown that fructose phosphorylation occurred 10 times more readily than glucose phosphorylation. Invert sugar which contains 50% fructose and 50% glucose, when injected into humans has been shown to produce less glycosuria than glucose(3,4). These differences suggest that fructose might be a better sugar for intravenous administration than glucose.

Present study. In each of 10 human subjects two comparative experiments were carried out. The control consisted of the intravenous infusion of 1 liter of 10% glucose. Previously or subsequently 1 liter of 10% fructose was injected at the same or a faster rate. Patients 1, 2, 3, 6 and 8 were patients in their late convalescent period of recovery from abdominal surgery and were relatively normal. The experiments on patients 4, 7, and 10 were performed on their second and third postoperative days and patient 9 was a mild diabetic not on insulin therapy. During and after each injection the urine passed dur-

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* This work was partially supported by grants from the Commonwealth Fund of New York, The Squibb Institute for Medical Research and Mead Johnson and Co.

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TABLE I. Comparative Effects of Intravenous Infusion of One Liter of 10% Glucose and of 10% Fructose.

Patient Age (yr) Sex Wt (kg)	Duration of infusion (hr)	Sugar infused	Whole blood glucose levels (mg %)				Glycosuria		Fructosuria		Diuresis		Infusion rate, g/kg/hr
			Initial	Middle	Final	Max. change	g/hr	total g	g/hr	total g	cc/hr	total cc	
1. G.T.	1.5	Glucose	61	295	73†	+234	1.6	10.3	0	0	70	455	1
50 M.	2	Fructose	89	88	81†	— 8	0	0	.2	1.4	26	185	1
64	.8	”	76	99	81†	+ 23	0	0	.6	3.8	37	26	2
2. C.W.	1.5	Glucose	70	260	57†	+203	1.2	8.2	0	0	80	520	1
62 M.	2	Fructose	70	110	101†	+ 40	0	0	.4	3.2	42	300	1
64	.8	”	109	163	138†	+ 54	0	0	.7	4.2	66	385	2
3. H.T.	1.8	Glucose	110	458	247†	+348	1.4	5.6	0	0	46	184	1
62 M	1.8	Fructose	110	126	118†	+ 16	0	0	.9	3.7	43	175	1
53													
4. F.M.	2.25	Glucose	112	334	450†	+338	4.4	17.7	0	0	200	690	.8
64 M.	2.25	Fructose	50	97	—	+ 47	0	0	.7	3.6	72	360	.8
62													
5. S.R.	2	Glucose	87	297	320*	+233	3.8	27.0	0	0	142	1000	1.1
67 F.	1.75	Fructose	90	106	98*	+ 16	0	0	1.1	5.2	155	700	1.2
44													
6. B.V.	1.75	Glucose	72	—	74‡	—	2.3	9.6	0	0	362	1450	.9
60 F.	1.25	Fructose	98	105	78‡	+ 7	0	0	.78	3.1	46	185	1.2
61													
7. J.D.	2.5	Glucose	97	320	334*	+237	1.8	21.4	0	0	43	500	.7
46 M.	2.08	Fructose	182	142	140*	— 42	0	0	.3	5.8	27	500	.8
57													
8. N.A.	2.5	Glucose	67	—	162*	—	.8	3.8	0	0	333	1510	.6
17 M.	2	Fructose	94	105	94*	+ 11	0	0	.2	1.0	172	860	.7
63													
9. E.H.	1.9	Glucose	218	316	342*	+124	4.1	24.9	0	0	380	1900	.7
50 F.	2	Fructose	180	236	216*	+ 56	0	0	.4	2.4	120	720	.7
79													
10. H.E.	2.5	Glucose	96	258	390*	+194	3.6	18.3	0	0	149	745	.8
40 F.	1.5	Fructose	91	112	96*	+ 21	0	0	.9	4.9	190	950	1.4
46													

* 30 min. post infusion.
† 60 min. post infusion.
‡ 120 min. post infusion

ing a period of several hours was collected and analyzed. Whole blood samples were taken at the beginning, at the mid period and at 30, 60 and 120 minutes after the end of the injection. Whole blood and urine glucose was determined by the method of Somogyi(5) and whole blood and urine fructose was determined by a modification of the method of Roe(6).

The 10% fructose solutions were prepared from C. P. fructose (Pfanstiehl) which was dissolved in pyrogen-free water, treated with Norit (activated charcoal), boiled, filtered, passed through a Berkefeld filter and then sterilized at 15 lb pressure for 20 minutes. Aliquots from each batch were analyzed for their fructose content.

Findings. The findings are shown in Tables I and II. It will be noted that the level of whole blood glucose which rose in a characteristic manner following the glucose injection, showed scarcely any increase following the fructose injection. The maximum change in the former was 348 mg % and in the latter case less than 60 mg % (Table I). On the other hand, when the fructose level of the blood was measured following the fructose injections, there was at most a moderate increase which returned almost to zero 60 minutes after the end of the infusion (Table II). Analysis of the urine showed remarkably low values of fructosuria which followed the fructose injection, in contrast to the frequently

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TABLE II. Effect of Intravenous Infusion of One Liter of 10% Fructose on the Level of Whole Blood Fructose.

Patient Age (yr) Sex Wt (kg)	Rate of infusion (g/kg/hr)	Whole blood fructose levels (mg%)		
		Initial	Middle	Final
1. G.T. 50 M. 64	1 2	0 0	41 102	6.5† 14 †
2. C.W. 62 M. 64	1 2	0 0	61 105	2.7† 5.6†
3. H.T. 62 M. 53	1	0	123	8.7†
4. F.M. 64 M. 62	.8	0	37	—
5. S.R. 67 F. 44	1.2	0	49	56 *
6. B.V. 60 F. 61	1.2	0	33	0 ‡
7. J.D. 46 M. 57	.8	0	38	65 *
8. N.A. 17 M. 63	.7	0	41	12 †
9. E.H. 50 F. 79	.7	0	46	11 †
10. H.E. 40 F. 46	1.4	0	76	40 *

* 30 min. post infusion.
† 60 min. post infusion.
‡ 120 min. post infusion.

high degrees of glycosuria which followed the glucose injection (Table I). The amount of sugar lost in the latter case was sometimes as much as 25% of that infused, whereas the maximum loss of fructose was 5.8%. The degree of diuresis which accompanied the glucose infusions was not seen following the fructose infusions. Of interest are the findings in the diabetic patient, No. 9. Also of interest are the findings in the patients tested during the immediate postoperative period, who showed a diminution of glucose tolerance.

Discussion. It seems apparent from the present findings that fructose when given intravenously to the human subject, even at rates as rapid as 2 g/kg/hr. leaves the circulation promptly. It does not appear in the urine and is presumably converted just as promptly into glycogen. These findings suggest that fructose is a much better sugar for intravenous feeding than glucose. Not only is less sugar and water lost in the urine but it is likely that the increases of hepatic glycogen which are undoubtedly obtained, have important practical and theoretical implications. For example, the ability of the liver to overcome harmful agents or stresses is undoubtedly increased thereby.

Summary. In 10 experiments on human subjects comparative intravenous injections of 10% glucose and 10% fructose were carried out. Even at rapid rates of infusion very little fructose appeared in the urine as compared to the amount of glucose. Diuresis was also less pronounced with fructose. Moreover, the fructose infusions were not followed by either hyperglycemia nor by an excessive rise in the blood fructose level, thus indicating a more rapid utilization of fructose and presumably more rapid glycogenesis.

Received November 21, 1950. P.S.E.B.M., 1950, v75.

Role of the Thyroid Gland in Urinary Pentose Excretion in the Rat.*

(18356)

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Coover, Feinberg, and Roe(1) found that the excretion of urinary pentose in rats was markedly increased by keeping the animals in

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* Supported by a grant from the Division of Research Grants and Fellowships, the National Institutes of Health, United States Public Health Service.

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TABLE I. Effect of Thiouracil Feeding on Urinary Excretion of Pentose. Data are in mg per 24 hr as arabinose.

Rat No.	Basal diet daily avg for 3 day period	Basal diet + 2% thiouracil. At room temp. (20°-30°C). Days 1	2	3	4	5	6	7	Basal diet only. Days 8	9
1	2.56	.74	.77	.15	.63	.77	.0	.0	1.41	1.89
2	2.48	.70	.84	.46	1.30	1.24	.97	1.95	.67	2.95
3	1.45	1.61	1.44	.55	.15	.95	.95	1.27	1.48	2.11
4	2.90	1.61	.63	.31	.70	.95	.62	.55	1.61	2.00
5	2.74	2.44	.18	.80	1.42	.15	.64	1.06	2.13	3.00
6	2.69	.23	.15	.08	.60	1.19	.81	.27	1.22	3.00
7	2.53	1.11	.78	.46	.80	.46	.75	.02	1.15	2.00
8	2.93	1.24	2.67	2.46	1.52	.93	.23	.30	.77	3.02
		Basal diet + 2% thiouracil at −1°C								
9	2.06	1.97	1.94	1.99	2.01	died				
10	1.95	.0	.0	died						
11	1.87	.24	died							
12	2.17	.14	died							
13	2.28	.0	died							
14	1.74	.08	.02	.0	died					

a cold room at −1°C and by thyroid feeding. It was suggested that the accelerated excretion of urinary pentose in the cold is due to increased metabolism brought about by greater thyroid activity.

Further evidence of the part played by the thyroid in the excretion of urinary pentose has been obtained by experiments in which thiouracil was fed to rats. The data are shown in Table I. Rats kept at room temperatures and placed on an adequate synthetic diet to which 2% of thiouracil was added showed a highly significant decrease in urinary pentose excretion ($P<0.01$). In some instances the daily urinary pentose excretion was less than the amount determinable by the method used (2) and is recorded as zero excretion. These observations appear to indicate that the thyroid gland has a regulatory influence upon the excretion of urinary pentose.

In the experiment of Table I in which rats were placed in the cold room at −1°C and fed a thiouracil-containing diet, the urinary excretion of pentose was markedly decreased, and the animals survived poorly. Of the 6 rats placed in the cold room, 3 died on the second day, and one died on the third, fourth and fifth days. Thus animals fed thiouracil cannot compensate sufficiently to survive at −1°C. This observation is further evidence for the assumption that the compensatory adjustment of animals to cold occurs through activity of the thyroid gland.

Summary. It has been observed in rats that thyroid feeding markedly increases the urinary excretion of pentose, and thiouracil feeding greatly reduces the output of urinary pentose. Animals on a diet containing thiouracil also survive poorly in the cold. These observations offer strong evidence for the assumption that in the rat the thyroid gland has a dominating role in the production of urinary pentose and that adjustment of animals to cold takes place, at least in part, through activity of the thyroid gland.

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Received November 21, 1950. P.S.E.B.M., 1950, v75.

Development of the Baculum of the Long-tailed Weasel.* (18357)

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It has been shown previously that the male long-tailed weasel *(Mustela frenata)* does not mature sexually until about one year of age. This is in spite of the fact that the animals often reach full body growth in captivity when 3 or 4 months old(1). The baculum remains small during this year of immaturity but develops to adult size when the animal first becomes sexually active. During the winter months when the males are 8 to 11 months of age they may be distinguished from the adults only by differences in the size and appearance of the baculum. The bacula of 16 immature males of *Mustela frenata oribasa* weighed from 14 to 29 mg and those from 18 adults 53 to 101 mg.

It seemed likely that the baculum is stimulated to develop to the adult type by androgens. In the immature hypophysectomized gonadectomized rat the baculum can be induced to develop by the injection of testosterone propionate(2). Experiments were planned to determine the effect of castration on both adult and immature weasel bacula and the effect of androgen treatment on the bacula of immature males.

Materials. Sixteen males were used in these experiments. Dr. Wm. H. Elder donated an entire litter obtained at Delta, Manitoba and 4 males from this litter were used. Four males, apparently littermates, from Kenosha, Wis., were used, and the additional animals were purchased from a trapper at Stevensville, Mont. All of the immature animals were caught during the summer when they can be recognized as immature by their undeveloped testes. The animals were maintained in individual cages and fed a diet consisting of horse meat, liver, and Purina mink chow.

TABLE I. Effect of Castration on Bacula of Adult and Immature Weasels.

Animal No.	Age of animal when castrated	Wt at autopsy, g	Date castrated	Date autopsied	Wt of baculum, mg
140	Adult	253	8/23/44	7/31/45	81.2
144	,,	260	4/29/45	7/31/45	59.4
158	Immature	269	12/31/45	7/ 6/46	26.0
159	,,	363	10/ 8/45	1/12/47	29.6

Results. The effect of castration on the size of the baculum was investigated as indicated in Table I.

It can be seen that the bacula of the adult males did not regress following castration; even though 11 months elapsed in one case (Fig. 1). This is to be expected since there is no regression of the baculum in normal adults during the winter months when the testes are regressed and androgen production is supposedly at a low ebb. That the immature males fail to develop the adult type baculum following castration when allowed to live considerably beyond the time in which they would have become adults, further suggests that androgens may be necessary for maturation of the baculum.

A preliminary experiment in which one immature male was injected with .5 mg testosterone propionate per day for 20 days indicated that the baculum was definitely under stimulation but did not develop fully in that time. Since a longer period of treatment was indicated and it is difficult to give daily injections to weasels without putting them under anesthetic, in further experiments pellets of androgen were implanted subcutaneously in the shoulder region. Such an experiment was carried out as indicated in Table II. At the end of the experiment the pellets were recovered, stripped of surrounding tissue, air-dried and weighed in order to determine the amount absorbed.

In only one of the 4 experimental animals

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* Aided by a Grant-in-Aid from Sigma Xi Research Fund. The pellets of testosterone propionate were kindly furnished by Schering Corporation.

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2. Thyberg, W. G., and Lyons, W. R., Proc. Soc. Exp. Biol. and Med., 1948, v69, 158.

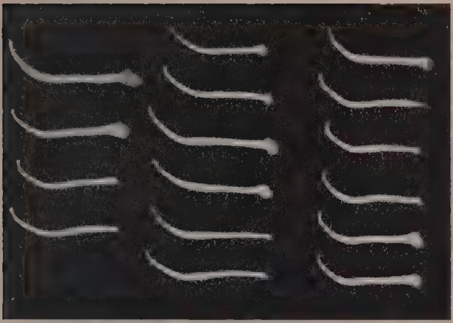

FIG. 1.
Bacula of the experimental animals (×⅔). The distal ends are at the left, the proximal ends at the right. The lateral corpora cavernosa are attached to the proximal end. The urethra lies in a sulcus on the ventral side of the bone. The group on the left show the bacula from Table I, #140 at the top. The group in the center are from Table II and the group at the right from Table III. In all cases the bacula are arranged in the order in which they are listed in the tables.

did the baculum develop to near the adult type. All of them, however, showed some development of the basal bulb of the baculum (Fig. 1). Since the desired effects were only partly realized, the experiment was repeated using testosterone propionate in place of testosterone. Two pellets were implanted per animal, and the animals were allowed to carry the pellets for a longer period. The testes of immature weasels are apparently completely inactive during the fall and winter months but in order to rule out any possible influence all the animals were castrated before the experiment was started.

In all cases the experimental animals developed larger bacula than did the controls. The weights and appearance of the bacula of these experimental animals closely approximate those of normal adults. They might have developed further with more prolonged treatment. Examination of the illustration (Fig. 1) clearly shows that the development of the basal nob is very marked. This seems to be correlated with a striking development of the corpora cavernosa which are attached to this nob. Histological sections of the penis made directly posterior to the baculum of these animals indicates that this portion of the penis is markedly stimulated by the androgen treatment, perhaps more extensively than the baculum.

Discussion. Lyons *et al.*(3) have shown that somatotrophin and androgen may synergize to produce a more marked effect on the os penis of the castrated hypophysectomized rat than either hormone alone. It is not known whether somatotrophin might have such an effect in the case of the weasel. Wild-caught adult male weasels average slightly heavier during the summer months than immature males(1). If an increase in body weight occurs at the same time as the onset of sexual maturity, as seems likely, it is possible that somatotrophin might have some minor effect upon the development of the baculum

TABLE II. Immature Weasels Implanted with 75 mg Pellets of Testosterone.

Animal No.	Body wt at autopsy, g	Date of implantation	Date of autopsy	Avg loss of wt of pellets, mg	Wt of baculum, mg
192	287	9/11/47	11/1/47	1.11	23.8
193	311	"	"	1.06	31.6
198*	366	"	"	0.93	50.2
199*	327	"	"	1.03†	31.6
200*	332	Control	"	—	30.0
201*	294	"	"	—	31.4

* No. 198-201 were littermates from Delta, Manitoba. These animals were considerably heavier than the animals from western Montana.

† Pellet was found to have fallen out on September 27, when it was replaced with another.

TABLE III. Immature Weasels Implanted with 2-75 mg Pellets of Testosterone Propionate. All animals castrated November 4, 1948.

Animal No.	Body wt at autopsy, g	Date of implantation	Date of autopsy	Avg loss of wt of pellets per day, mg	Wt of baculum, mg
219	301	12/1/48	2/1/49	0.61	47.4
226	287	Control	"	—	27.4
230*	234	"	"	—	22.8
231*	268	"	"	—	31.4
232*	229	12/1/48	"	0.96	43.6
233*	219	"	"	1.08	39.6

*230-233 were probably littermates from Kenosha, Wis. These animals were slightly smaller than those from western Montana and considerably smaller than those from Manitoba used in the previous experiment.

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3. Lyons, W. R., Abernathy, E., and Gropper, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 193.

in the weasel. Petrides(4) has recently discussed the problem of aging various fur bearing mammals by such characters as the baculum and the value of obtaining data of this type in the management of these species. If the results of this study do apply to other closely related species such as the marten, fisher, wolverine, badger, etc. whose sexual cycles are not fully understood, it would mean that those animals in the wild with adult type bacula were animals which had been sexually mature during the previous breeding season. This would be of value in estimating age ratios of these animals taken by trappers.

Summary. The bacula of castrated immature male long-tailed weasels developed to the adult type after the animals were implanted with pellets of testosterone propionate. The bacula of untreated castrated immature males did not develop to the adult type after several months. Once the baculum develops to the adult type it does not regress after castration.

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Received November 22, 1950. P.S.E.B.M., 1950, v75.

Renal Function in Decompensated Cirrhosis of the Liver.* (18358)

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Patients with decompensated cirrhosis of the liver have, as is well known, an impaired capacity for excreting sodium(1-5). Studies of glomerular filtration rate and renal plasma flow have, however, been few and the data obtained contradictory (Table I). The present study was intended to expand existing information and report simultaneous observations on glomerular filtration rate and electrolyte excretion, with a view toward drawing some conclusions concerning the mechanism of salt and water retention in cirrhosis of the liver and to correlate the degree of liver im-

TABLE I. Summary of Inulin, Mannitol, and Para-aminohippurate Clearances in Patients with Cirrhosis of the Liver Reported from Various Laboratories.*

Author	No. of subjects	Accumulating fluid	Presence of ascites	Inulin clearance, ml/min.	Mannitol clearance, ml/min.	PAH clearance, ml/min.
Patek(6)	1	Yes	Yes	91		434
	1	"	"	142		600
	1	"	"	172		709
Leslie(7)	5	"	"	69†		371
	2	No	"	136†		704
	6	"	No	201†		846
Farnsworth(1)	1	Yes	Yes		61	415
	1	"	"		74	476
Goodyer(5)	1	"	"		60	298
	1	"	"		88	449
	1	"	"		94	526
	1	"	"		102	492
	1	"	"		123	418
	1	"	"		124	647

* The data of Patek and Goodyer are corrected to 1.73 sq.m., the data of Leslie and Farnsworth apparently are not corrected for surface area.
† Mean.

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* This work was supported in part by the New York Heart Association.

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3. Eisenmenger, W. J., Ahrens, E. H., Jr., Blondheim, S. H., and Kunkel, H. G., *J. Lab. Clin. Med.*, 1949, v34, 1029.

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5. Goodyer, A. V. N., Relman, A. S., Lawrason, F. D., and Epstein, F. H., *J. Clin. Invest.*, 1950, v29, 973.

6. Patek, A. J., Jr., Mankin, H., Colcher, H., Lowell, A., and Earle, D. P., Jr., *J. Clin. Invest.*, 1948, v27, 135.

7. Leslie, S. H., Johnson, B., and Ralli, E. P., *J. Clin. Endocrin.*, 1949, v9, 682.

TABLE II. Clinical and Laboratory Data on Patients with Cirrhosis of the Liver.

Name	Age, yr	Sex	Wt gain per wk, lb.†	Inulin clearance,* ml/min.	PAH clearance,* ml/min.	Plasma Na, mEq/min.	Urinary Na excr.,† mEq/min.
T	51	M	6	93	705	131.4	1.5
K	59	F	2	98	324	135.6	88.0
WS	50	M	9	103	565	127.8	0.7
H	46	M	13	105	425	139.7	2.6
F	41	F	7	106	700	141.6	13.7
G	67	M	7	127	488	138.4	1.9
NS	53	M	8	127	710	135.2	58.0
NK	58	F	4	132	521	—	89.0
BW	46	M	8	132	610	140.6	90.0
WT	36	M	0‡	141	660	136.7	70.0
BG	54	M	8	141	618	132.4	1.5
O	34	M	4	169	1110	144.0	12.0

* Avg of 3 consecutive collection periods corrected to body surface area of 1.73 Sq.M.

† The wt gain per wk cannot be correlated with the urinary sodium excretion since the dietary sodium intake was not strictly controlled in all patients.

‡ WT was undergoing a spontaneous diuresis at the time of measurement.

pairment with renal function.

Methodology. Twelve patients suffering from Laennec's cirrhosis of the liver confirmed by history and by clinical findings were studied. All showed diminished liver function on the basis of cephalin flocculation, thymol turbidity, bromsulfalein retention, and plasma albumin level. Subjects were selected to exclude those with findings indicative of cardiac or renal disease. All patients had required abdominal paracentesis during a period of illness which ranged from 3 to 60 months (average 16 months). All had ascites and edema of varying extent; 11 were accumulating fluid and one (WT) was undergoing a spontaneous diuresis at the time of measurement (Table II). The patients were ambulatory in the hospital. They received a high protein, high caloric diet containing at least 3 to 5 g (50 to 80 mEq) of sodium chloride per day. Mercurial diuretics and abdominal paracenteses were withheld for several days prior to study. Glomerular filtration rate was measured as the inulin clearance and renal plasma flow was measured as the p-aminohippurate clearance. After an appropriate priming dose, 5% glucose containing inulin and sodium p-aminohippurate was administered intravenously at a rate of 1 ml per minute. This solution was freshly prepared and the glucose did not remain in contact with the p-aminohippurate longer than 3 hours(8). If a reaction product formed between the two, the blood flows would be still higher than the reported values. Urine was collected in periods of 20 to 30 minutes through an indwelling catheter. Venous blood was collected at the mid-point of each urine collection period. All clearance data are reported as the mean of 3 consecutive collection periods. Inulin was measured in urine and plasma by Harrison's modification(9) of Alving's method(10). p-Aminohippurate was measured in diluted urine and trichloroacetic acid filtrates of plasma by the Bratton-Marshall reaction(11). Sodium was determined with an internal standard flame photometer (12).

Observations. The inulin clearance varied from 93 to 169 ml/min. which is within the normal range of published data(13). The mean for all subjects was 123 ml/min. (Table

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8. Baldwin, D. S., Schreiner, G. E., Breed, E. S., Wesson, L. G., Jr., and Maxwell, M. H., *J. Clin. Invest.*, 1950, v29, 614.

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13. Smith, H. W., *Lectures on the Kidney*. University Extension Division, University of Kansas. Lawrence, Kansas, 1945.

II). Likewise, the individual clearances of p-aminohippurate fell within normal limits apart from the lowest (324 ml/min.) and the highest (1110 ml/min.). The mean renal plasma flow was 595 ml/min.

Plasma sodium values were normal except for 3 patients. In view of a normal filtration rate the load of sodium presented to the tubule can be considered essentially normal. The actual urinary excretion of sodium, however, was practically negligible in 5 patients (1 to 2 micro equivalents/min.), while the others excreted sodium in amounts which could be considered normal. It is evident, therefore, that the inability to excrete sodium in cirrhosis is not a function of the filtration rate.

The degree of abnormality of the liver function tests was not related to the rate at which the patient was accumulating fluid. Moreover, in the same patient spontaneous diuresis may occur prior to any significant change in the liver function tests, as was subsequently observed in patients WS and WT. This confirms the well known clinical observation that the degree of liver damage measured by the usual tests does not necessarily parallel salt retention.

Comment. The data presented demonstrate that in cirrhosis of the liver profound salt retention can occur in the presence of a normal glomerular filtration rate. It is not doubted that for a given patient the capacity to eliminate sodium will be greater at higher than at lower rates of filtration(14) but this does not imply that a low filtration rate is the cause of sodium retention. The present data indicate that sodium retention in cirrhosis of the liver is due to excessive tubular reabsorption since a normal sodium load is presented to the tubule. This observation is in accord with the findings of Goodyer and his group(5). In addition, they were able to demonstrate that patients with cirrhosis still failed to eliminate sodium in the normal fashion even though the sodium load was increased by the intravenous infusion of 5% saline.

Summary. Glomerular filtration and renal plasma flow were found to be normal in eleven patients with cirrhosis of the liver who were accumulating edema fluid indicating that the retention of sodium is due to increased tubular reabsorption.

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Received October 31, 1950. P.S.E.B.M., 1950, v75.

Compensatory Hypertrophy of the Intact Adrenal of Fetal Rats Subjected to Unilateral Adrenalectomy.* (18359)

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There are several lines of evidence that the mammalian adrenal begins to function prior to birth. The suprarenal cortex of the human fetus becomes markedly enlarged(1). Obviously this is only circumstantial evidence, and, as has been pointed out(2), the physiological significance of this hypertrophy is not understood. It has been demonstrated that pregnancy prolongs the life of adrenalectomized dogs(3,4). The removal of the adrenals of pregnant rats causes an increase in the weight of the adrenals of the fetuses (5,6). The removal of the hypophysis of fetal rats by decapitation inhibits the growth of the adrenal(7,8), as does also the destruction of the hypophysis of fetal mice by ir-

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* Aided by grants from (1) the Committee on Human Reproduction, National Research Council, acting on behalf of the National Committee on Maternal Health and (2) The Ciba Pharmaceutical Products, Inc.

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2. Windle, W. F., Physiology of the Fetus, W. B. Saunders Co., 1940, xiii + 249.

radiation(9). This effect has been prevented experimentally by introducing adrenocorticotrophin under the skin of headless fetuses(10, 11). In fetal rats in which the adrenals had been destroyed by applying cautery through the translucent uterine wall, Tobin reported a decrease in the number of acidophilic cells of the hypophysis and an absence of effects upon the reproductive tract(12). The implantation of a pellet of androgen beneath the skin of the fetal rat increased the volume of the fetal adrenal(13). On the other hand, there is some evidence that the adrenals of fetal rats do not produce an androgenic hormone. In adrenalectomized fetuses, those changes in the seminal vesicles which can be induced by castrating fetuses were not observed(14).

It is generally accepted that the removal of one adrenal after birth causes a compensatory hypertrophy of the remaining gland(15-18). Thus it was decided to subject fetal rats to unilateral adrenalectomy, to autopsy them before birth and to make a quantitative study of the remaining adrenal. Hypertrophy of this remaining organ would suggest that the adrenal begins to function before birth in that it is already able to respond to an experimentally introduced stimulus.

Procedure. The rats used were of the Sprague-Dawley strain. The estimated age of the fetuses was dated from the time of witnessed copulation. The litter was used as the experimental unit. At approximately 19 days and 11 hours after copulation, a mother was etherized and a fetus was presented for surgery by removing it from the uterus without interrupting the placental circulation(19,20). Using a binocular dissecting microscope, iridectomy scissors and jeweler's forceps, an oblique lumbar incision was extended into the abdominal cavity of the fetus and the left adrenal was removed. After suturing the abdominal incision, the fetus was placed in the maternal peritoneal cavity and the etherization of the mother was discontinued. At the end of the experimental period the fetus was obtained by sacrificing the mother at about 21 days and 15 hours after copulation (at about one hour preceding the estimated time of earliest parturition)(20). The operated fetus and a littermate control of the same sex were weighed, killed and eviscerated in order to expose their adrenals. The eviscerated bodies were fixed in Bouin's solution. After fixation the adrenal of both fetuses was dissected, removed, dehydrated, imbedded in paraffin and sectioned in series at 10 micra. The sections were stained with hematoxylin and eosin. Throughout this entire procedure, the glands of a litter were subjected, as closely as possible, to the same conditions. The volume of an adrenal was determined from the stained serial sections by the paper-weight method of Hammar(21). Every sixth section was projected at a magnification of 50 diameters and drawn. Then the formula of Boyden(22) was adapted and used.

Observations. The volumetric data are presented in Table I. Not shown is the fact

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TABLE I. Volume of the Right Adrenal.

Groups	No. of fetuses	Age at operation, da : hr	Exper. period, hr	Body wt, g	Vol., $mm^3 \cdot 10^2$	Vol., $mm^3 \cdot 10^2$ / Body wt, g	
						Per group	E/C
Control[*]	6			4.54(3.89-5.20)	37.8(24.47-52.45)	8.3(5.60-10.20)	
Exper.[†]	7	19:11(19:9-19:13)	53(51-55)	4.23(3.66-4.86)	50.5(33.73-62.75)	11.9(9.21-14.79)	1.43(1.08-1.65)

[*] Seven observations in 6 fetuses (3 male and 3 female).

[†] Seven fetuses from 6 litters (3 male and 4 female).

that the "t test" was applied to those data from which the averages in the third vertical column from the right were obtained. In making this test, the first step was to determine the difference between the experimental and control values, litter by litter, by making a series of seven simple subtractions (23). The outcome suggests that these differences are "highly significant" (probability of t = .001).

Comparable sections of the glands were microscopically studied with the aid of a microcomparator, an instrument which brings one-half the field of each of two microscopes into view. When a section of adrenal of an experimental fetus (section E) was thus directly compared with that of a litter mate control (section C), it was usually found that the capsule in E was thinner than in C. This suggests that it had been "stretched" by the "pressure of growth" of the cortex. An outer zone of cortical cells, comparable in position to the zona glomerulosa of postnatal life, was usually thinnest in E. The cells of this zone were largest in E. This increase involved both the cytoplasm and the nucleus. An intermediate zone in C was characterized by vacuolated cells; this zone was widest in E and its cells were largest in E. In E this intermediate zone appeared to have increased in width at the expense of cells from both the outer and inner zones. In an inner zone, the thickness was approximately the same in E and C, but the cells were the largest and contained the most vacuoles in E.[†] The vascular sinusoids were most conspicuous in E, suggesting that the blood supply of the gland had been increased. This method of observation failed to reveal any apparent differences in mitotic activity between C and E. There were no marked differences between the section of a male adrenal and that of a female adrenal, in either C or E.

Discussion. The observations support most of those of Tobin(12), and extend them. He studied a remaining adrenal of each of 3 fetuses in which he had been unsuccessful in performing a bilateral adrenalectomy by means of cautery. He reported a 15 to 20% increase in $\frac{\text{volume}}{\text{body wt}}$ (cf. an increase of 43% in our series). My material fails to confirm his report of an increased thickness of the "glomerulosa zone."

The observations presented are in agreement with those of Swinyard and Bruner(17). In dogs unilaterally adrenalectomized after birth, they noted an increased size of the intact gland and attributed this to an increase in size of its cortical cells.

The hypertrophy observed is new evidence that the fetal adrenal is able to respond to an introduced stimulus. However, it fails to assist in answering the question as to whether the functioning of the adrenals of fetuses is a factor in prolonging the life of animals adrenalectomized during pregnancy. This effect was attributed to the fetal adrenal by Billman and Engel(4). Rogoff and Stewart(3) suggested that the maternal ovary and corpus luteum were factors since they failed to observe hypertrophy of the adrenals of newborn puppies from mothers adrenalectomized during the period of gestation. Recent studies have brought out evidence which supports the view that either the corpus luteum(24) or proges-

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23. Treolar, A. E., Random Sampling Distributions, Burgess Publishing Co., Minneapolis, 94 p., 1942.

[†] The developing adrenal medulla, like that of the human fetus, is not distinctly circumscribed, but instead consists of cellular nests which give the impression of lying in the fetal cortex.

terone(25) will prolong the life of certain adrenalectomized mammals including the ferret and the cat. Regardless of what prolongs the life of adrenalectomized pregnant females, it has been shown that adrenalectomizing the mother results in hypertrophy of the adrenals of fetuses(5,6). It has also been found that the injection of adrenal cortical extracts into the mother will prevent these effects(6). These observations raise a question as to whether the same factors responsible for hypertrophy of fetal adrenals after adrenalectomizing the mother play a part in the compensatory hypertrophy noted in the unilaterally adrenalectomized fetuses of this present series. It is reasonable to assume that one factor in both these instances is the action of an adrenocorticotrophin. A number of recent observations point to the view that adrenocorticotrophin is produced by the hypophysis of the mammalian fetus(7-12). Against this view is a recent report that in immature rats subjected to injections of epinephrine or to cold the hypophysis does not release ACTH until the 8th day and 16th day, respectively, after birth(26). This report poses a question as to whether the effective adrenocorticotrophin might have come from the maternal hypophysis, the fetal hypophysis or, conceivably, the placenta. Also one has to consider the possibility of the direct effect of reduced quantities of adrenal cortical hormones upon the fetal adrenal. From the observations noted in this series, it is not possible to settle the matter.

Conclusion. Subjection of the fetal rat to unilateral adrenalectomy causes a compensatory hypertrophy of the intact adrenal. Manifestations of this include an increased volume of the gland and an increased size of cells of all cortical zones. Those cells at both borders of the intermediate zone show an increased vacuolation, suggesting that the zone had been widened by the metamorphosis of cells at these borders.

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Received November 2, 1950. P.S.E.B.M., 1950, v75.

Arteriovenous Shunts in the Human Lung.* (18360)

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The presence of arteriovenous (A-V) shunts bypassing the capillary circulation in the human lung has been suspected from studies on blood gas analyses(1), the passage of certain parasites through the lung(2), and the formation of telangectasis(2) and arteriovenous aneurysms(3-6) within the lung.

Although these shunts have not been observed in the human lung(7,8), Prinzmetal *et al.*(9) have shown that glass spheres many

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* This study was supported in part by the Fluid Research Fund of the University of Rochester School of Medicine and Dentistry, and by the Eastman Kodak Company, Rochester, N. Y.

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6. Lindskog, G. E., Liebow, A., Kausel, H., and Janzen, A., *Ann. Surg.,* 1950, v132, 591.

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times the accepted diameter of capillaries in all species will pass through the pulmonary vessels of the dog and rabbit. Experiments were therefore undertaken, utilizing the glass sphere technic followed by injection media, to ascertain the presence and anatomical location of A-V shunts in the human lung.

Material and methods. Fresh human lungs (obtained at autopsy from 20 adults, 17-91 years of age, and 3 newborn infants) showing little or no evidence of respiratory pathology, were used. The lungs were gradually inflated, via the bronchus, with compressed air (5-10 mm Hg pressure) to approximately normal size and then allowed to deflate by their inherent elasticity. Glass cannulae were ligated in the hilar ends of the pulmonary artery and veins, and normal saline solution perfused (30-40 mm Hg pressure) into the pulmonary artery to wash out the residual blood. Perfusion was continued until a relatively clear perfusate returned from the pulmonary veins. Several hundred small glass spheres (10-750 μ in diameter)[†] were then poured into a small glass funnel, connected by rubber tubing to the cannula in the pulmonary artery, and washed into the artery with 50-100 cc of saline solution. The rubber tubing was clamped with a hemostat, the funnel removed, and the outer surface of the lung washed thoroughly to remove any spheres that might possibly have fallen there. The cannulated specimen was placed in a clean white-enameled basin, the hemostat removed, and 500 cc of saline perfused through the pulmonary artery at 50-300 mm Hg pressure. The outer surface of the lung was again washed with saline to collect any spheres that might have adhered to the lung after leaving the cannulated pulmonary veins. The perfusate returning from the venous cannulae and the washings collected in the basin were decanted and filtered. Recovery of the spheres could be enhanced by examining the basin or filter paper under ultraviolet light, since the spheres fluoresce with a different color from that of the enameled basin or the filter paper. The spheres recovered were counted, and their sizes measured on a stage micrometer. Solutions of liquid latex or vinyl acetate[‡] were injected into the pulmonary vessels at an initial pressure of 60-100 mm Hg, which was increased to 200-250 mm Hg as peripheral resistance to the injection masses was encountered. Acetone perfusion preceded the vinyl acetate injection. The pulmonary arteries were injected with red, and the pulmonary veins with blue colors of these solutions. Radiopaque media[§] were added to the injection mass used in 6 adult lungs, to permit radiographic visualization of the pulmonary vessels and localization of the spheres. The lungs injected with liquid latex were embalmed by perfusing 10% formalin into the bronchi; those injected with vinyl acetate were macerated in concentrated hydrochloric acid to obtain casts of the vessels.

Observations. Spheres up to 500 μ in diameter were washed through the pulmonary veins from 9 of the adult and 2 of the neonatal lungs by the saline perfusion, with an average of 37.5 spheres recovered from each lung. No spheres were recovered in the perfusate from the other 12 lungs. However, when the pulmonary veins of these lungs were dissected (if injected with latex) or dissolved in acetone (if filled with vinyl acetate) they were

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9. Prinzmetal, M., Ornitz, E. M., Jr., Simkin, B., and Bergman, H. C., *Am. J. Physiol.*, 1948, v152, 48.

[†] Some of the glass spheres were obtained through the kindness of Dr. Myron Prinzmetal, Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif., others were purchased from B. F. Drakenfeld, 45-47 Park Place, N. Y. City.

[‡] Stock solutions of the liquid latex and vinyl acetate, in various colors, were purchased from Ward's Natural Science Establishment, Rochester, N. Y. The injection syringe consisted of a glass cylinder and a metal piston with a rubber washer at the end of the piston, such as the Janet-Frank Bladder Syringe or the B-D Champion Veterinary Syringe. This type of syringe is necessary, because latex or vinyl acetate will cause metal to adhere to metal or glass to glass.

[§] The radiopaque injection media consisted of: vinyl acetate 90% and 10% iodoform; liquid latex 75% and 25% of a saturated solution of barium sulphate in a 25% ammonium hydroxide; or the liquid latex and 25% stabilized colloidal thorium dioxide contrast medium (Thorotrast, manufactured by the Heyden Chemical Corporation, New York City); or liquid latex and 25% ethyl iodophenylundecylic acid (Pantopaque).

found to contain spheres up to 500 μ in diameter. Spheres larger than 500 μ were lodged in the arteries and did not pass to the veins.

The anatomical location of the A-V shunts was ascertained from radiographs of the lungs injected with radiopaque media, in which the course of the vessels and the spheres themselves could be visualized; from dissections of the latex-injected lungs; and from the casts of the specimens injected with vinyl acetate. No A-V shunts were present in the hilar region nor along the course of the larger branches of the pulmonary artery and vein. At the apex of the lobular subdivision of the bronchopulmonary segments, however, shunts up to 500 μ in diameter were found. Smaller shunts (50-100 μ) were located at the level of the smaller bronchi and respiratory bronchioles, and the smallest, (20-25 μ) near the alveolar sacs and the alveoli. Shunts up to 200 μ in diameter also occurred in the pleura.

Comment. Perhaps a larger number of spheres and a higher percentage of successful passages of spheres through the pulmonary veins might have been found if gelatin, or some other solution of greater viscosity than the saline perfusate, had been used, since many of the spheres settled in the more dependent parts of the arteries and veins. Such viscous solutions, however, would have interfered with the subsequent injection of latex or vinyl acetate. Unless the A-V shunts were relatively large, they could not be demonstrated by the vinyl acetate technic, apparently due to the shrinkage and fixation of the vessels by the pre-injection acetone perfusion or the acetone content (approximately 80%) of the vinyl acetate and the small percentage (less than 20) of vinyl resin remaining after the specimen was macerated.

Summary. Arteriovenous shunts, many times the accepted diameter of capillaries, were demonstrated in isolated human lungs with little or no pulmonary pathology, by the passage of glass spheres from the pulmonary artery to or through the pulmonary veins. Injection of these vessels with liquid latex or vinyl acetate indicates that the shunts are located at the apex of and within the lobular divisions of the lungs.

Received November 27, 1950. P.S.E.B.M., 1950, v75.

Antigenic Pattern of Strains of Influenza A and B. (18361)

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Studies on the antigenic pattern of influenza A and B viruses have revealed heterogeneity in each of these groups(1-5). The significance of this multiformity as it relates to the occurrence and recurrence of strains of a particular antigenic composition in the human population and to the selection of viruses for use in the vaccine has not been fully evaluated. The present communication presents data on the antigenic patterns of a number of strains of influenza A and B which have been recovered since 1933.

Materials and methods. Virus strains. Certain pertinent information about the viruses used in the present work is summarized in Table I. Additional data are given in Table II on the strains designated CAW-1, CAW-2, CAW-3, CAW-4A, CAW-5, Canadian Arctic 1A and Canadian Arctic 12A which were recovered from specimens collected from Es-

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* Colonel, M.C., U.S.A.

† Captain, M.C., U.S.A.

1. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, v19, 273.

2. Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, v19, 284.

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4. Gordon, I., *J. Immunol.*, 1942, v44, 231.

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TABLE I. Data on Strains of Influenza A and B Included in Studies.

Type	Strain	Year isolated	Passage history	Origin
A	WS	1933	F?/M?/>E5	England
’’	PR8 (ISSC)*	34	F1/M691/E7-8	Puerto Rico
’’	Hyde	35	F?/M?/>E9	Probably Alaska
’’	Henry	36-37	M32/E4	N. Y.
’’	Hickcox	40-41	F1/M12/E4	N. Y.
’’	Weiss	43	M79/E6-7	Mich.
’’	FM1 (ISSC)*	47	M7/E15-17	N. J.
’’	Hume	47	E7	Md.
’’	Wilfong	48	E21-22	Calif.
’’	4ML-1-48/49	48-49	>E2	Heidelberg, Germany
’’	Canadian Arctic 1A	49	E3	Victoria Island, Can
’’	Canadian Arctic 12A	49	E3	’’ ’’ ’’
’’	CAW-1	49	E3	’’ ’’ ’’
’’	CAW-2	49	E3	’’ ’’ ’’
’’	CAW-3	49	E3	’’ ’’ ’’
’’	CAW-4A	49	E3	’’ ’’ ’’
’’	CAW-5	49	E3	’’ ’’ ’’
’’	SF-1-49	49	E6-7	Calif.
’’	AH-1-50	50	E2-3	Ga.
’’	Berkeley-1-50	50	F1/E5	Calif.
’’	FW-1-50	50	E3	Wyoming
B	Lee (ISSC)*	40	F8/M310/E7-8	N. Y.
’’	Hawaii	44-45	>E3	Hawaii
’’	Czech	45	E6-7	Mass.
’’	Warner	48	>E4	Australia
’’	Seattle-2-49	49	E8	Washington
’’	Gt. Lakes 34	50	E6-8	Ill.
’’	Gt. Lakes 38	50	E5	’’
’’	Gt. Lakes 40	50	E4-5	’’
’’	IB1	50	E7	N. Y.
’’	L1-B-50	50	E8	N. J.
’’	MB	50	E6-9	N. Y.
’’	Meguro	50	E7-8	Japan
’’	POOH	50	E14-16	’’

F = Ferret, M = Mouse, E = Egg.

* Standard diagnostic strains of the Influenza Strain Study Center, Commission on Influenza, Armed Forces Epidemiological Board.

TABLE II. Data on Strains from Victoria Island Outbreak, April 1949.

Strain	Isolated by	Isolated from patient		Material
		Ref. #6	Ref. #7	
CAW-1	Nagler	B.T.	Too	Nasopharyngeal wash.
CAW-2	’’	M.C.	Car	’’ ’’
CAW-3	’’	A.W.	Wil	’’ ’’
CAW-4A	’’	B.P.	—	Heart blood-autopsy
CAW-5	’’	M.M.†	Meg‡	Lung autopsy
C.A.* 1A	van Rooyen	—	Ban	Nasopharyngeal wash.
C.A.* 12A	’’ ’’	—	Meg‡	Lung autopsy

* C.A. = Canadian Arctic.

† M.M. of Table II (not Table I) in reference 6.

‡ Strains CAW-5 and C.A. 12A were isolated in independent laboratories from lung material of patient Meg (M.M.).

This table compiled from information furnished by Drs. F. P. Nagler and C. E. van Rooyen.

kimos on Victoria Island, N.W.T., Canada during April 1949(6,7). The epidemic in the Arctic was characterized(6) as "an unusually virulent outbreak of influenza, reminiscent of the 1918 pandemic" in which there was an attack rate of 100% and case fatality rate of

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6. Nagler, F. P., van Rooyen, C. E., and Sturdy, J. H., *Canad. J. Pub. Health,* 1949, v40, 457.

7. van Rooyen, C. E., McClelland, L., and Campbell, E. K., *Canad. J. Pub. Health,* 1949, v40, 447.

20% in the 90 Eskimo inhabitants of the island. In several instances, the same patients and materials were given different designations by Dr. F. P. Nagler and Dr. C. E. van Rooyen. Only two of the strains in the present study, *i.e.*, CAW-5 and Canadian Arctic 12A, were recovered from the same patient (M.M. or Meg.).

Hyde and Hume viruses(8) were furnished by Dr. B. Eddy. The Henry and Hickcox strains were obtained from Dr. T. P. Magill and the Berkeley-1-50, from Dr. G. Meikeljohn. The sources of the remaining A type strains used in the present study were given in an earlier report(9). It may be mentioned that our FW-1-50 strain(9) is sometimes designated Cuppett by other workers. The Lee and Czech strains of influenza B virus were furnished by Dr. T. P. Magill and the L1-B-50 and Seattle-2-49 by Drs. M. M. Sigel and A. S. Lazarus, respectively. The Meguro and POOH viruses, isolated at the 406th Medical General Laboratory, Tokyo, Japan, were sent by Major K. F. Burns, V. C., U.S.A. and Dr. F. L. Horsfall supplied the MB and IBI viruses. The Warner agent was obtained from Dr. S. G. Anderson and the Hawaii strain, isolated at the 18th Medical General Laboratory, Honolulu, Hawaii, was from the strain files of this department. The Great Lakes 34, 38 and 40 viruses, from a recent outbreak of influenza B at the Great Lakes Naval Training Station, Illinois, were furnished by Commander John R. Seal, M.C., U.S.N.

Sera. In general, pairs of roosters weighing 5 to 8 lb were injected on one occasion by the intravenous and intraperitoneal routes with 5.0 ml amounts of crude infected allantoic fluid which had been freshly harvested. However, birds receiving MB, IBI, POOH, L1-B-50 or Seattle-2-49 viruses were inoculated with these volumes on 2 successive days since it was found that the single inoculation failed to evoke a satisfactory antibody response to these agents. The roosters were bled 10 days following the initial injection and their sera stored in lyophilized form until used in the tests. Sera against strains IBI, Warner, POOH, L1-B-50 and SF-1-49 titered 1:400 with homologous antigen; all other sera titered 1:800 or greater. Sera obtained from roosters prior to immunization did not contain demonstrable antibody against influenza virus.

Hemagglutination technic. The hemagglutination and hemagglutination-inhibition titrations were performed by a technic which conformed to the standard diagnostic method recommended by the Committee on Serological Procedure of the Armed Forces Epidemiological Board(10). In the latter procedure, 0.25 ml volumes of serial dilutions of serum were mixed with 0.25 ml of virus (4 units) and 0.5 ml of 0.5% human type "O" red cell suspension. The pattern of sedimented cells was read after 50 minutes and the titer taken as the highest initial dilution of serum giving complete inhibition of hemagglutination. The antigens were preserved by lyophilization and rehydrated within 24 hours of their use in the tests. In the tables, the serum titers were adjusted to give a 1:800 titer with homologous virus so that the results with the individual sera could be compared more easily.

Results. Influenza A. Tables III and IV summarize the results of tests with strains of influenza A. Table III gives the numerical values for certain of the serum titers summarized in interpretive form in Table IV. In the latter table, a titer of 1:800 is represented by 4+, 1:400 by 3+, 1:200 by 2+, 1:100 by 1+ and 1:50 by ±. In 3 instances the adjusted serum titer obtained in tests with heterologous antigen was 1:1600, *i.e.*, 2 times as high with homologous antigen. This difference was not considered significant, and for the purpose of simplification, this titer was represented as 1:800 (4+) in the table. The values given in the tables were obtained in tests with single serum specimens. Essentially identical cross-reactions were obtained in those instances in which viral anti-

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8. Ward, T. G., and Eddy, B. E., *Science*, 1950, v112, 501.

9. Hilleman, M. R., Mason, R. P., and Rogers, N. G., *Public Health Repts.*, 1950, v65, 771.

10. Committee on Standard Serological Procedures in Influenza Studies, *J. Immunol.*, 1950, v65, 347.

TABLE III. Numerical Summary of Agglutination-Inhibition Tests with Representative A Type Influenza Strains.

Rooster antisera	Serum titers obtained with antigens						
	WS (1933)	CAW-3 (1949)	PR8 (1934)	FM1 (1947)	CAW-5 (1949)	Wilfong (1948)	FW-1-50 (1950)
WS 1933	*800**	0	400	50	200	0	0
CAW-3 '49	0	*800*	800	0	50	0	0
PR8 '34	0	200	*800*	0	0	0	0
FM1 '47	0	0	50	*800*	800	800	50
CAW-5 '49	ND	0	100	800	*800*	800	100
Wilfong '48	0	50	100	400	800	*800*	200
FW-1-50 '50	0	0	50	400	800	800	*800*

* All titers expressed as reciprocals of fractions.
ND = Not done.

TABLE IV. Interpretive Summary of Agglutination-Inhibition Tests Showing Relationships of Influenza A Strains.

Rooster antisera	Antigens												
	WS 1933	CAW-3 1949	PR8-ISSC 1934	Weiss 1943	Henry 1936-37	Hickcox 1940-41	Hyde 1935	Hume 1947	FM1 1947	CAW-5 1949	Wilfong 1948	SF-1-49 1949	FW-1-50 1950
WS 1933	*4+*	0	3+	2+	±	1+	0	0	±	2+	0	0	0
CAW-3 '49	0	*4+*	4+	2+	1+	±	1+	1+	0	±	0	0	0
PR8-ISSC '34	0	2+	*4+*	2+	4+	±	3+	2+	0	0	0	0	0
Weiss '43	0	1+	2+	*4+*	2+	1+	2+	1+	0	0	0	0	0
Henry '36-7	±	2+	3+	3+	*4+*	2+	4+	3+	1+	2+	+	0	±
Hickcox '40-1	2+	1+	4+	4+	3+	*4+*	4+	3+	2+	3+	2+	1+	±
Hyde '35	0	±	1+	2+	4+	2+	*4+*	3+	1+	1+	1+	0	0
Hume '47	0	±	1+	3+	3+	3+	4+	*4+*	1+	2+	3+	0	0
FM1 '47	0	0	±	2+	3+	3+	4+	3+	*4+*	4+	4+	±	±
CAW-5 '49	ND	0	1+	ND	ND	ND	ND	ND	4+	*4+*	4+	2+	1+
Wilfong '48	0	±	1+	2+	2+	2+	2+	2+	3+	4+	*4+*	2+	2+
SF-1-49 '49	0	0	±	0	1+	1+	1+	1+	4+	4+	3+	*4+*	3+
FW-1-50 '50	0	0	±	0	2+	2+	2+	0	3+	4+	4+	4+	*4+*

ND = Not done.

gens were tested with the antisera obtained from the other chicken of the pair which received a given virus. Thus, it would appear that the qualitative antigenic response of different birds to a particular virus was relatively consistent. Similar results were obtained with materials from certain of the strains examined; in most instances such reduplicative detail has been omitted from the tables. Thus, the CAW-1, CAW-2, CAW-3, CAW-4A and Canadian Arctic 1A viruses were indistinguishable from each other; hence, only data on CAW-3 are presented in detail. CAW-5 and Canadian Arctic 12A agents were essentially identical. Strain 4ML-1-48/49 could not be differentiated from SF-1-49 and the AH-1-50 and Berkeley-1-50 viruses did not differ from FW-1-50. The present hemagglutination-inhibition tests with chicken antisera revealed wide antigenic differences among the strains of influenza A examined. While some were essentially identical, such as those mentioned above as well as the Hyde and Hume strains listed in Table IV, others, *i.e.*, WS and FW-1-50, had no demonstrable relationship to each other. Although differences between the more distantly related strains were easily recognized, the precise segregation of the majority of the A viruses into sharply demarcated subgroups was rendered impossible by the existence of a graded series of intermediate forms.

Influenza B. Table V gives an interpretive summary of the findings with strains of in-

TABLE V. Interpretive Summary of Agglutination-Inhibition Tests Showing Relationships of Influenza B Strains.

	Antigens												
Rooster antisera	Lee 1940	Gt. L. 40 1950	Gt. L. 38 1950	Gt. L. 34 1950	Hawaii 1944-45	Czech. 1945	MB 1950	IB1 1950	Warner 1948	Meguro 1950	POOH 1950	L1-B-50 1950	Seat-2-49 1949
Lee 1940	4+	3+	3+	3+	1+	±	±	0	±	1+	1+	±	1+
Gt. Lakes 40 '50	4+	4+	3+	3+	1+	1+	1+	±	±	1+	1+	1+	2+
Gt. Lakes 38 '50	4+	4+	4+	4+	1+	2+	±	±	1+	1+	1+	1+	2+
Hawaii '44-45	2+	1+	±	±	4+	3+	2+	1+	1+	3+	2+	3+	2+
Czech. '45	3+	1+	1+	1+	4+	4+	3+	1+	2+	2+	1+	3+	3+
MB '50	1+	1+	1+	±	3+	2+	4+	1+	±	2+	1+	3+	2+
IB1 '50	2+	2+	2+	2+	4+	4+	4+	4+	3+	4+	4+	4+	4+
Warner '48	0	0	0	0	4+	3+	3+	3+	4+	4+	4+	3+	3+
Meguro '50	0	0	0	0	2+	2+	2+	3+	3+	4+	4+	3+	3+
POOH '50	0	0	0	0	4+	4+	3+	3+	4+	4+	4+	3+	3+
L1-B-50 '50	2+	0	0	0	4+	3+	3+	2+	2+	4+	3+	4+	3+

fluenza B. It is apparent that these agents fell into 3 general groups with Lee, Hawaii and Warner viruses serving as the prototypes. It is noteworthy that the B viruses studied did not display the numerous intermediate variations encountered among the A viruses examined by this method. This difference may be due more to chance selection of strains than to a real difference between the 2 groups; perhaps other intermediate forms will be found among B strains.

Discussion. Present studies on the antigenic composition of influenza viruses isolated prior to 1945 are, for the most part, limited to strains which have undergone numerous passages in animals and eggs. Since such treatment may induce significant alteration in antigenic pattern(11,12), our knowledge of the constitution of these viruses as they originally occurred in the human population and of their native relationships to each other, must remain largely theoretical.

The question of whether influenza viruses exist in nature in constant or changing antigenic form has been speculated upon by a number of workers(3,9,13,14). Some evidence for the latter possibility was presented in an earlier report(9) in which it was shown that certain strains of influenza A, isolated in the United States and Europe since 1947, showed progressive change in antigenic pattern in successive years. These tests were repeated in the present studies with essentially identical results giving credence to the idea of continuing change in influenza A. A possible explanation for the origin of such variants was suggested by Taylor(14) who conceived that natural passage of influenza virus in the human subject, where antibodies are encountered as a result of previous infections, may give rise to strain deviation.

It is of importance to note, however, that irrespective of whether a single line or several lines of influenza virus exist in nature in constant or changing form, strains of virus close in antigenic composition to earlier prototypes have recurred after widely separated time intervals. Thus, the CAW-3 virus, which was similar to PR8 (Table IV), occurred 15 years following the isolation of this latter agent. Likewise, the Hyde and Hume viruses which were essentially identical, were isolated 12 years apart. The similarity of Hyde and Hume strains to FM1 in hemagglutinin tests with FM1 chicken antiserum was in contrast to the findings of Ward and Eddy(8) who detected no relationship by the mouse neutralization test. Likewise, the failure of PR8 antiserum to react significantly with WS virus, as shown in this and other studies employing hemagglutination(15,16), was not demon-

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11. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 357.

12. Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 143.

13. Magill, T. P., and Sugg, J. Y., PROC. SOC. EXP. BIOL. AND MED., 1943, v53, 104.

14. Taylor, R. M., *Am. J. Pub. Health*, 1949, v39, 171.

strated by the mouse test(8); with the latter technique, WS virus was strongly neutralized by PR8 antiserum. Such observations as these point up the different results obtained in certain instances by the two methods and the hazards of relying on one method alone for evaluating the relationships among strains of influenza virus.

The data (Table IV) concerning the CAW and Canadian Arctic viruses from the outbreak among Eskimos on remote Victoria Island, N.W.T., are of special interest. In the first place, 2 widely different antigenic forms were apparently involved in the outbreak: CAW-3 virus resembled PR8 and CAW-5 was similar to FM1. While strains resembling PR8 have not been found for a number of years, viruses of the same general form as FM1 were isolated from patients throughout the populated cities in the Dominion during the winter of 1949(7). In the second place, these viruses, which were antigenically similar to recognized laboratory strains, were apparently responsible for this outbreak in which the morbidity and case fatality (100% and 20% respectively) approximated that of the last great pandemic. Either virus was presumably of sufficient virulence to cause death, directly or indirectly, since strains CAW-4A (resembling PR8) and CAW-5 (resembling FM1) were recovered from autopsy material. The excessive morbidity in this outbreak may have been due to lack of recent experience with influenza virus as indicated by the low antibody titers in the Eskimos of the region prior to the epidemic(7). Many of the deaths were probably due to secondary bacterial invaders since the sulfa drugs and penicillin were highly effective in treatment(6). It is of considerable interest that these viruses which produced a limited outbreak of disease of high morbidity and mortality among Eskimos were not of unusual virulence for the 50-odd white inhabitants of the region. The latter, who were probably immunized through earlier experience with influenza virus, did not suffer serious infection at the time; indeed, only 1 required medication.

The diversity in antigenic constitution of influenza viruses necessitates the inclusion of a plurality of strains in the prophylactic vaccine to provide a complete coverage against agents from the earlier as well as more recent outbreaks of the disease. Spatial and practical considerations limit the number of strains which can be incorporated into the vaccine so that it is desirable to select a group of strains in which most of the principal antigens of influenza viruses are represented and which are satisfactory in other respects such as growing abundantly in eggs, eliciting a good immunologic response in man and being antigenically stable on inactivation and storage.

Present formula influenza vaccine for military and civilian use is made of PR8 (22 2/9%), FM1 (22 2/9%), FW-1-50 (also called Cuppett or Z virus—22 2/9%) and Lee (33 1/3%) viruses(17). The data in Table IV revealed that while most strains of influenza A appeared to be covered by antisera against the viruses in the vaccine, a definite antigenic deficiency was suggested in that none of the antisera reacted significantly with WS. In view of the broad antigenic pattern of Hickcox virus, in the current tests, further studies on this A strain appear warranted. The present vaccine also appears to be deficient in its coverage of B strains. The present work, as well as that of others(4,5, 18,19) showed that the Lee strain is appreciably different from a number of other B viruses. The latter shortcoming might be eliminated by incorporation of 1 of the more recently recovered strains of B virus such as IBI.

The 1233 strain of Taylor(14) together with the same or similar agent recently iso-

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15. Hudson, N. P., Sigel, M. M., and Markham, F. S., *J. Exp. Med.*, 1943, v77, 467.

16. Hirst, G. K, *J. Exp. Med.*, 1943, v78, 407.

17. Laboratory of Biologics Control, National Institutes of Health, Memorandum to Manufacturers of Influenza Virus Vaccines, dated October 11, 1950.

18. Lazarus, A. S., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 317.

19. National Office of Vital Statistics, Public Health Service, Communicable Disease Summary for Week Ended May 6, 1950, Laboratory Supplement of May 11, 1950.

lated by Francis, Quilligan and Minuse(20) poses another problem as regards the current vaccine. Whether these will eventually be included as immunizing agents remains to be determined, but at the moment the question is essentially a theoretical one since the new strains are difficult to grow by the methods commonly employed for commercial production of influenza virus.

While the selection of strains of influenza virus for use in a vaccine cannot be based on *in vitro* studies alone, the agglutination-inhibition test might be expected to assist materially in the choice of new strains for detailed examinations. The marked strain specificity of chicken antisera when employed in the influenza agglutination-inhibition test appears to provide a simple and effective means for the rapid preliminary antigenic survey of a large number of strains.

Summary. Influenza A viruses, as revealed by agglutination-inhibition tests with rooster antisera, comprised a graded series of antigenic forms in which precise subgrouping was not possible by the present method. The B strains examined fell into 3 subgroups. The reappearance from time to time of strains closely related to an earlier prototype, necessitates the selection of a group of strains for a polyvalent vaccine which possess broad immunogenic activity. Certain possible deficiencies in the present formula vaccine were mentioned. The recent outbreak of influenza among Eskimos in remote Victoria Island, Canada, in which there was mortality and morbidity approaching the pandemic form of disease, was apparently caused by 2 different viruses which resembled the well-studied PR8 and FM1 strains of influenza A.

The technical assistance of SFC F. Hawk and SFC W. O. Blair is gratefully acknowledged.

Received November 20, 1950. P.S.E.B.M., 1950, v75.

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Enhancing Effect of Cortisone upon Poliomyelitis Infection (Strain MEF1) in Hamsters and Mice. (18362)

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It was recently shown that ACTH and cortisone were capable of inhibiting the phenomenon of local tissue reactivity. The substances failed to suppress the state of reactivity following the preparatory injection of the bacterial toxin but conferred protection against the effect of the provocative intravenous injection of the toxin(1,2). These findings were confirmed by Thomas and Mogabgab. In extending the studies they found that when the preparatory intradermal injection of the toxin was accompanied by an intramuscular injection of ACTH or cortisone, there appeared a petechial reaction at the site of the toxin injection in the absence of the provocative intravenous injection of the toxin. In rabbits receiving no cortisone or ACTH the intradermal injection of toxin failed to produce any hemorrhagic reaction unless followed by a provocative injection of the toxin. The lesions described differed sharply in appearance from the typical reaction of the phenomenon of local tissue reactivity(3). Since the observations suggested that ACTH and cortisone may be capable of enhancing the susceptibility of tissues to the primary effect of bacterial toxins, they were led to determine the effect of the hormones upon bacterial infections. In experiments in this direction Mogabgab and Thomas

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observed a marked enhancement of blood and tissue infection by *Streptococcus hemolyticus* in rabbits following injection of cortisone(4). The appearance of a bacteremia during crisis in a case of pneumonia treated with ACTH by Finland, Kass and Ingbar is also of interest in this connection(5).*

Taking into consideration the above findings and the fact that pregnant women in whom there is a greatly enhanced adrenocortical function(6), show an increased incidence of poliomyelitis(7), the author of this paper carried out the following studies on the effect of ACTH and cortisone upon experimental poliomyelitis infection.

Experimental. Strain MEF1 kindly supplied by Dr. Peter K. Olitsky was employed in these studies. The virus was maintained in this laboratory by serial intracerebral mouse passages. In the course of the work the identity of the virus was checked by neutralization tests with anti-Lansing monkey serum, received from the Lederle Laboratories through the courtesy of Dr. Hilary Koprowski. Swiss male mice weighing 10-14 g and male hamsters weighing 22-27 g were used in the experiments described. Only mouse brain passages were employed for inoculation of mice and hamsters. The intracerebral inoculation dose for both animal species was 0.05 ml of the brain emulsion, suitably diluted with saline. All surviving animals were examined daily for a period of one month. In a large control series carried out previously in this laboratory in connection with other studies, the hamsters were found quite resistant to poliomyelitis infection. Intracerebral inoculation of MEF1 strain gave rise to a mild disease of irregular onset, low incidence, and very low mortality rate. Thus, out of 61 hamsters 10 became paralyzed 4-19 days following inoculation with MEF1 mouse brain emulsion diluted 1 : 20. Three paralyzed hamsters died several days later, while 7 survived during the entire period of observation. The remaining 51 hamsters had no symptoms and showed normal weight gain. The above observations confirm the previous studies of Stebbins and Lensen(8).

The experimental groups and the accompanying control series of animals recorded in Table I were inoculated with the same brain emulsions. Several experimental hamsters and mice were killed at various stages of the disease. The brain, liver, spleen, and blood were cultured on a variety of aerobic and anaerobic media, and the brain emulsions diluted 1:100 were inoculated intracerebrally into mice in order to ascertain the presence of poliomyelitis virus. With the exception of a few surface contaminations of brain and liver (*i.e.*, *Staphylococcus albus* and *E. coli*), the sacrificed animals showed no evidence of bacterial infection. The emulsions of the brains removed from the experimental animals produced typical poliomyelitis in mice. ACTH and cortisone were administered intramuscularly, each dose being 5 mg. When 3 injections were made, the intervals of time were 4 and 19 hours. The results of the studies were as follows:

In the first series of experiments 10 mice receiving one injection of ACTH and two injections of cortisone and 10 untreated mice were each inoculated intraperitoneally with 0.5 ml of MEF1 mouse brain emulsion diluted

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TABLE I. Effect of ACTH and Cortisone Upon Experimental Poliomyelitis.

				Intracerebral inoc. of MEF1		Deaths without paralysis		Animals paralyzed		Deaths following paralysis			
Group No.	Animal species	Total No. of animals	Treatment	Dose, dil.	Time interval between treatment and inoc.	No.	Days after inoc.	No.	Days after inoc.	No.	Days after inoc.	No. survivors with paralysis	No. survivors showing no symptoms
1	Mouse	9	—	1:100	—	—	—	7	8-12	6	9-11	1	2
2	”	9	ACTH 1x Cortisone 2x	1:100	2 hr after ACTH	—	—	9	2-4	7	3-8	2	—
3	Hamster	21	—	1:20	—	—	—	5	4-6	2	6-7	3	16
4	”	21	ACTH 1x Cortisone 2x	1:20	2 hr after ACTH	5	3-4	16	4-7	16	5-8	—	—
5	”	10	Cortisone 3x	1:20	2 hr after 1st injection Cortisone	—	—	10	2-5	10	4-6	—	—
6	”	10	Cortisone 1x	1:20	Simultaneously with Cortisone	—	—	10	2-6	10	3-8	—	—
7	”	10	ACTH 3x	1:20	2 hr after 1st inj. ACTH	—	—	4	3-6	1	6	3	6
8	”	10	—	1:100	—	—	—	3	7	—	—	3	7
9	”	10	ACTH 1x Cortisone 2x	1:100	2 hr after ACTH	—	—	10	2-6	10	4-8	—	—

1:10. In the treated group, the inoculation of the virus was made 2 hours after the injection of ACTH. Two mice of the treated group died 8 days following virus inoculation, having shown no symptoms of poliomyelitis, while 8 mice of the treated group and all 10 mice of the untreated group remained well during the entire period of observation. Thus, the hormones failed to induce in mice susceptibility to parenteral inoculation of MEF1 strain of poliomyelitis virus.

The results of the second series of experiments in which all the virus inoculations were made intracerebrally are summarized in Table I.

The mice recorded in Table I were inoculated with MEF1 brain emulsion diluted 1:100 in order to obtain a delayed infection. The control group (Group 1) developed the disease 8-12 days following inoculation. There was observed in mice treated with ACTH and cortisone (Group 2) a marked shortening of the incubation period, paralysis appearing 2-4 days following inoculation with a correspondingly earlier mortality. Thus, the experiments indicate a marked acceleration of the infection in a susceptible host upon combined administration of ACTH and cortisone. The results assume, however, a much greater significance when viewed in the light of the following findings in hamsters. The administration of the hormones induced a prompt and violent infection. Within a day or two after inoculation the treated hamsters appeared very weak and drowsy. They showed swollen eyelids, conjunctivitis, ruffled fur, and hunched backs. The paralysis often occurred 2 days following inoculation, and was quite extensive involving all extremities of some animals. The disease was uniformly fatal. As may be seen from Table I, all 51 hamsters of groups 4, 5, 6, and 9 treated with ACTH and cortisone or cortisone alone died 3-8 days following the virus inoculation. While 5 died having shown no paralysis, the remaining 46 hamsters developed paralysis 1-3 days prior to death. When these results are compared with the controls recorded in Table I (Group 3 and 8) and the control series mentioned in the text, it becomes obvious that the hormones are capable of inducing an extraordinary enhancement of susceptibility to the experimental poliomyelitis infection in the hamster. The hormonal effect transforms a mild infection into a rapidly progressing, violent and uniformly fatal disease, and eliminates completely the natural refractoriness of the animal.

In the initial experiment both ACTH and cortisone were used. On further study it was found that cortisone alone was necessary for the effect, a single intramuscular injection of cortisone administered simultaneously with the intracranial inoculation of the virus sufficing for the production of an equally marked enhancement of susceptibility to the infection, (Groups 5 and 6). It may be noted, however, that hamsters which received 3 injections of ACTH alone in a total dose as large as 15 mg, (Group 7) failed to show enhanced susceptibility to the infection. These results suggest the possibility that in addition to cortisone, ACTH stimulates also the secretion of another factor which may be capable of reversing the enhancing effect of cortisone.

The following control series of experiments were done in order to ascertain that the results obtained were not due to enhancement by the treatment with the hormones of some latent animal virus, rather than to the enhancement of susceptibility to the poliomyelitis virus inoculated. The materials tested were obtained as follows:

1. Two mice received one injection of ACTH and two injections of cortisone at time intervals used in the experimental series. The brains were removed 2 hours after the second injection of cortisone; 2. brains of two normal mice, and 3. two mice were injected intracerebrally with 0.05 ml of broth diluted 1:10 with saline. The brains were removed 24 hours later.

Each brain emulsion diluted 1:10 with saline was inoculated intracerebrally into 10 untreated mice, 10 untreated hamsters, and 5 mice and 10 hamsters which received one injection of cortisone simultaneously with the inoculation. All animals remained well during the entire period of observation.

Summary and Conclusions. ACTH and cortisone in combination or cortisone alone produce a marked acceleration of poliomyelitis infection (strain MEF1) in mice and an extraordinary enhancement of susceptibility to this infection in hamsters giving rise to a violent and uniformly fatal disease. ACTH alone fails to produce this effect possibly due to elaboration of an unknown factor capable of reversing the enhancing effect of cortisone. The experiments seem to indicate the existence of a significant relation between adrenocortical function and susceptibility of mice and hamsters to experimental poliomyelitis.

Capable assistance of Miss Alice Fisher and Miss Doreen Hollings is gratefully acknowledged.

Received November 27, 1950. P.S.E.B.M., 1950, v75.

Effect of Succinate on Contractility and Phosphocreatine and Adenosine Triphosphate Content of the Dog Heart.* (18363)

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The oxidation of succinate is catalyzed in cardiac muscle with great rapidity. Addition of succinate in substrate quantities to excised cardiac muscle causes a striking acceleration of oxygen consumption, both at high(1-3) and at low(4-5) oxygen tensions. On this

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* This work was supported by a grant from the Life Insurance Medical Research Fund.

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TABLE I. Effect of Succinate on the Heart-Lung Preparation of the Dog.

	Succinate*										
Dog	Dose, g	Duration of inj., min.	Rate of inj., g/min.	Heart rate, min.$^{-1}$	Mean systemic arterial pressure, mm Hg	Pulmonary arterial pressure, mm H_2O	Right atrial pressure, mm H_2O	Systemic output, cc/min.	Coronary output,† cc/min.	Total output, cc/min.	Work, kg/m/min.
67	0	0	0	120	93	—	34	312	—	—	—
	1.97	10	.197	116	91	—	41	292	—	—	—
69	0	0	0	176	105	255	10	466	42	508	.851
	1.03	3	.344	156	101	229	39	282	35	317	.504
70	0	0	0	168	104	173	19	486	42	528	.833
	1.54	10.5	.147	156	105	168	19	489	39	528	.836
76	0	0	0	156	106	186	34	415	75	490	.792
	1.80	14	.129	144	106	164	34	430	69	499	.795
77	0	0	0	164	106	136	25	449	91	540	.844
	1.29	2	.645	156	104	150	47	329	102	431	.655
202	0	0	0	148	118	188	46	577	70	647	1.151
	1.93	4	.483	140	115	173	61	459	45	504	.868
205	0	0	0	174	110	191	47	444	102	546	.915
	1.71	6	.285	162	107	168	60	332	157	489	.740

* As unhydrated anion.
† Coronary sinus outflow × 10/6.

ground the use of the 4-carbon compound has been advocated as a possible therapeutic measure in myocardial weakness and anoxia (6). However, when the compound is administered to isolated heart preparations, the contractile power of the muscle is seen to diminish (7-9). A possible clue to this behavior is provided by the finding of Furchtgott and Shorr(5) that phosphorylation in cardiac muscle slices is depressed by succinate. The experiments reported below indicate that this may also be the case in the intact beating heart.

Methods. Starling heart-lung preparations of dogs weighing 8.1 to 12.0 kg were used. The weight of the hearts ranged from 51 to 75 g. The amount of circulating blood was approximately 800 cc, making the weight of the entire heart-lung system approximately 1 kilogram. The preparations were set up and their activity was measured as specified in a previous report(10). Succinate was injected as a 10% aqueous solution of the hexahydrated disodium salt over periods ranging from 2 to 14 minutes into the tube connecting the venous reservoir with the heart. The quantities injected are given in grams of the unhydrated anion, 0.43 g of which are equivalent to 1 g of the hydrated salt. Within one minute after the end of the injection a piece from the apex of the left ventricle was cut off and immediately immersed into liquid nitrogen. The frozen muscle was analyzed for adenosine triphosphate (ATP), phosphocreatine, inorganic phosphate, and total acid-soluble phosphate according to procedures outlined or referred to previously(10).

Results. Effect on Cardiac Function. The effect of succinate on the activity of seven heart-lung preparations is shown in Table I. The cardiodynamic measurements were taken shortly before the administration of the compound and then again at the completion of the administration, immediately before a sample of the heart was taken for the analysis of the phosphate compounds. In the majority of the experiments the hearts went into failure when succinate was injected, as indicated by

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TABLE II. Concentration of Some Acid-soluble Phosphates in the Hearts of Dog Heart-Lung Preparations Following Administration of Succinate.*

The figures in the italic print lie outside the fiducial limits of the respective control values (mean ± S.D. x t) for P = 0.05.

Dog	Time of survival, min.	Degree of heart failure‡	P, mg per 100 g ventricle			
			Inorganic phosphate	Phosphocreatine	Labile P of ATP	Total acid-soluble phosphate
8 controls,						
Mean†	82.6	—	28.15	14.33	35.21	104.10
± S.D.			±3.51	±1.64	±2.66	±6.47
67	152	Slight	25.9	*10.1*	31.9	97.5
69	138	Severe	32.7	*9.5*	*28.8*	94.4
70	67	None	33.4	*8.6*	30.1	100.7
76	67	,,	28.5	11.9	35.2	100.0
77	77	Severe	*36.6*	*4.4*	32.7	106.1
202	72	Moderate	30.6	*8.9*	*25.0*	*87.0*
205	124	,,	28.2	14.0	30.7	101.0

* For amounts of succinate administered, see Table I.

† For individual values, see (10).

‡ For details, see Table I.

a rise of the right atrial pressure and by a fall of the systemic arterial pressure, minute output, and work. However, the succinate had to be supplied to the heart at a fast rate in order to make it fail. The compound was without effect on myocardial contractility when administered at rates of 0.129 and 0.147 g per minute (hearts No. 76 and 70). Slight failure ensued at a rate of administration of 0.197 g per minute (heart 67). In the other experiments, in which the rate of administration was 0.285 g per minute and higher, the ensuing heart failure was fairly severe, although the total amount given was not much larger or was even smaller than in the experiments in which failure was absent or slight. That the changes observed were entirely due to the succinate anion is evidenced by the fact that administration of equivalent amounts of sodium chloride had not the slightest effect. A slight decrease in heart rate by succinate was observed in all preparations. Pulmonary arterial pressure was likewise slightly decreased. The effect on the coronary circulation was variable and apparently unrelated to the intensity of the inotropic effect.

Effect on Phosphocreatine and ATP Content. In all hearts, with one exception (No. 205), succinate produced a fall of the phosphocreatine level (Table II). Comparison with figures obtained in 8 control experiments shows that according to the statistical criterion selected in Table II this fall was of significant magnitude in 5 of the 7 succinate experiments, the phosphocreatine values lying from 30 to 67% below the mean of the controls. The concentration of ATP was significantly decreased in 2 hearts. A close parallelism between the impairment of contractility and the depletion of phosphocreatine or of ATP is not apparent from the data. The phosphocreatine was low in one of the 2 hearts in which contractility remained unimpaired. In heart 202 the total acid-soluble phosphate was low, but this does not account for its low content of phosphocreatine and ATP. For even if the phosphate of these 2 compounds is expressed in percent of the total acid-soluble phosphate, the figures remain below the lower fiducial limit of the respective control values.

Effect on Hearts Poisoned with Anesthetics. In 1943 Soskin and Taubenhaus(11) reported that succinate counteracts depression of central nervous system activity by pentobarbital. Subsequently, however, the usefulness of succinate as an antidote to barbiturates was questioned by others and became the subject of considerable controversy in the pharmacological literature. Since pentobarbital and other anesthetics are also capable of depres-

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TABLE III. Effect of Succinate on a Heart-Lung Preparation Poisoned with Pentobarbital.

Time of survival, min.	Compound admin.	Heart rate, $min.^{-1}$	Mean systemic arterial pressure, mm Hg	Pulmonary arterial pressure, mm H_2O	Right atrial pressure, mm H_2O	Systemic output, cc/min.	Coronary output,* cc/min.	Total output, cc/min.	Work, kg/m/min.	Competence index
67		156	106	262	18	420	87	507	.859	.87
71	0.07 g pentobarbital sodium									
80		144	102	274	40	303	130	433	.715	.57
91-95	0.64 g succinate									
96-97		140	97	269	53	185	114	299	.472	.38

* Coronary sinus outflow × 10/6.

TABLE IV. Effect of Succinate on a Heart-Lung Preparation Poisoned with Chlorobutanol.

Time of survival min.	Compound	Heart rate, $min.^{-1}$	Mean systemic arterial pressure, mm Hg	Right atrial pressure, mm H_2O	Systemic output, cc × $min.^{-1}$	Competence index
23		148	113	29	485	.92
25	0.15 g chlorobutanol					
52		144	113	50	385	.49
53	0.64 g succinate					
69		148	110	71	260	.25
71	0.64 g succinate					
73		154	106	86	155	—
81		156	109	76	215	.00

sing the activity of the heart(12), it seemed of interest to examine the effect of succinate on hearts poisoned with these agents. Table III summarizes an experiment in which 0.64 g succinate was given to a heart-lung preparation failing as a result of previous administration of pentobarbital. In addition to the cardiodynamic variables listed in Table I the response of the right atrial pressure to an increase in blood supply was determined in this experiment as a sensitive measure of its competence(13), and the results are expressed as the competence index(10). It is seen that succinate aggravated the heart failure caused by pentobarbital. The work done diminished by 34 percent, and the competence index which had been lowered by the barbiturate from 0.87 to 0.57, fell to a value of 0.38. In heart failure produced in another heart-lung preparation by the anesthetic chlorobutanol, succinate likewise exerted a strong negative inotropic action (Table IV). Again a dose of 0.64 g of succinate caused further impairment of contractility, and shortly following injection of a second dose of 0.64 g 18 minutes later the heart was in a state of very severe failure. After 10 more minutes had elapsed, it had slightly recovered from the impact of the succinate injection, but was still unable to handle an increase in load, as reflected in a competence index of zero. To judge from the data in Table I, the administration of 0.64 g succinate over a period of more than 4 to 5 minutes to a non-failing heart probably would have been without effect on its contractile power. It appears that poisoning the heart with an anesthetic makes it more susceptible to the negative inotropic action of succinate.

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Discussion. Although the heart in the heart-lung preparation is well provided with oxygen, its phosphocreatine store becomes depleted in the presence of large amounts of succinate, and it may go into failure. The decrease in phosphocreatine distinguishes this failure from that occurring in this preparation as the result of spontaneous deterioration or of poisoning with anesthetics(10). The present data do not constitute proof that succinate heart failure is actually due to depletion of energy-rich phosphate, particularly since the ATP content was found to be only slightly diminished and a close correlation between the decrease in contractility and in phosphocreatine was not apparent. However, they at least are suggestive of such a mechanism.

An explanation for the loss of phosphocreatine in the heart supplied with large quantities of succinate may be furnished on the basis of existing information about the coupling of succinate oxidation with phosphorylation. The occurrence of this coupling has been demonstrated in extracts of heart muscle and other tissues(14-17). However, the efficiency of phosphorylation linked to succinate oxidation is low. According to Ochoa(17) no more than one phosphate bond is formed per atom of oxygen taken up. Using whole heart homogenates Potter(18) was unable to obtain a net uptake of inorganic phosphate with creatine as phosphate acceptor when succinate was the substrate. In slices of dog myocardium incubated in 20% oxygen Furchtgott and Shorr(5) found that substrate quantities of succinate, while greatly stimulating oxygen consumption, depressed phosphorylation and produced a fall in the concentration of phosphocreatine and ATP. The succinate apparently inhibited the oxidation of endogenous substrates and was itself oxidized for the most part only to fumarate. The authors conclude that the energy furnished in this step was largely or completely wasted, the number of phosphate bonds formed per molecule succinate oxidized being much below the value of 1 postulated for heart extracts(19). In view of the fact that succinate had the same effect in the heart-lung preparation as it did in Furchtgott and Shorr's study in cardiac slices, namely, a lowering of the energy-rich phosphate concentration in the myocardium, it is quite likely that their conclusions are likewise applicable, at least in a qualitative fashion, to the intact beating heart. At any rate, the heart-lung experiments indicate, in agreement with earlier observations on substrate-depleted myocardium(9,20), that succinate is a poor source of energy for the contraction of the heart.

Of further interest in this connection is the finding that poisoning the heart with pentobarbital or chlorobutanol seems to make it more susceptible to the negative inotropic action of succinate. It had been shown previously(3) that on addition of succinate to cardiac slices whose respiration is severely depressed by pentobarbital or chlorobutanol, the oxygen uptake rises to the same level as in unpoisoned slices, and that for a considerable period almost the total oxygen uptake can be accounted for by the oxidation of the succinate to fumarate. While there is evidence (10) that heart failure produced in the heart-lung preparation by these anesthetics is not due to impairment of oxidative metabolism, there is, nevertheless, reason to believe(cf. 21) that in this failure the oxidation of substrates normally available to the heart, such as glucose and lactate, is inhibited to some extent. If inferences may be drawn from the behavior of heart slices with respect to the situation obtaining in the intact beating organ, it would appear that when succinate is administered to hearts poisoned with these anesthetics, the

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succinic oxidase system, the activity of which is not affected by these agents, is able to compete even more successfully with other oxidation systems for oxygen than in unpoisoned hearts, with the result that the negative inotropic action of succinate becomes more intense and prolonged.

Summary. When succinate is administered to the heart-lung preparation of the dog at sufficiently high rates (0.2 g or more of unhydrated succinate anion per kg heart-lung system per minute) the phosphocreatine concentration in the myocardium declines markedly and the heart goes into failure. The concentration of ATP declines to a lesser extent or remains unchanged. Heart failure in the heart-lung preparation caused by pentobarbital and chlorobutanol is intensified by succinate. These findings are discussed in the light of existing information on the coupling of succinate oxidation with phosphorylation and on the metabolism of cardiac tissue poisoned with anesthetics.

Received November 14, 1950. P.S.E.B.M., 1950, v75.

Amino Acids of the Shope Papilloma Virus. (18364)

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Among the symptoms attributable to viral infections is the production of tumors, such as those, for example, of infectious papillomatosis (Shope papilloma) of rabbits(1). Interest in the Shope papilloma virus is heightened by the possibility that findings gained from studies of it may, in addition to contributing to knowledge of host-virus relationships, possess implications for the cancer problem(2). A desirable basis for further studies on rabbit papillomatosis is provided by investigation of the physical and chemical nature of the agent inducing the disease. Progress in this direction has been made by Beard and associates who, from studies made on purified preparations, have reported the virus to be composed of desoxypentose nucleic acid, protein, and perhaps a small amount of lipid(3-8).

In the present investigation, several purified preparations of papilloma virus have been obtained, and the 2 largest of these have been analyzed for amino acid content by means of microbiological methods of assay. It was not possible to perform so exhaustive an analysis as was done with some strains of tobacco mosaic virus(9), owing to the relatively greater difficulty in procuring substantial quantities of highly purified virus; nevertheless, this report is presented with the hope that the data given may guide others working or proposing to work in this field.

Methods and Findings. Preparation of virus for assay. The source of virus for the experiments described herein was the warty growths removed from cottontail rabbits trapped in Kansas. The papillomas, which had been stored in glycerin-saline solution(4) upon harvest were drained on a fluted filter in a room held at 4°C, and then freed of hair and connective tissue. The naked warts thus obtained were deposited in a previously weighed amount of Sorenson's M/15 phosphate buffer at pH 6.6. A final amount of buffer was added to each lot of warts to give about a 20% suspension. The warts were then ground for 5 minutes in a Waring blendor at room temperature. The mash thus obtained was allowed to stand overnight at 4°C and then was spun in an angle centrifuge for 10 minutes at 5,000 R.P.M. The supernatant fluid was saved and the angle pellets were resuspended in buffer, and ground in the blendor for 5 minutes. The pellets from this low-speed centrifugation were discarded, and the supernatant fluid was pooled with the first lot. The combined fluids were spun once more in the angle centrifuge and were then spun at 25,000 R.P.M. (about 60,000 g) for 1 hour. The pellets obtained from the high-speed run were taken up in buffer solution at pH 6.6 and spun in the angle centrifuge at 5,000 R.P.M. for 10 minutes.[†] The brown pellets obtained upon angle centrifugation were discarded, and the supernatant fluid, containing the virus, was returned to the high-speed centrifuge. This process of alternate low- and high-speed centrifugation was repeated 4 to 5 times to yield clear gelatinous pellets of virus. The buffer concentration was progressively reduced, and the final pellets were taken up in distilled water from which the material was obtained in a white fluffy form by drying in the frozen state. Before analysis the final product was further dried to constant weight *in vacuo* over P_2O_5 at 65°C.

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* This investigation was begun in the laboratories of the Rockefeller Institute for Medical Research, Princeton, N. J.

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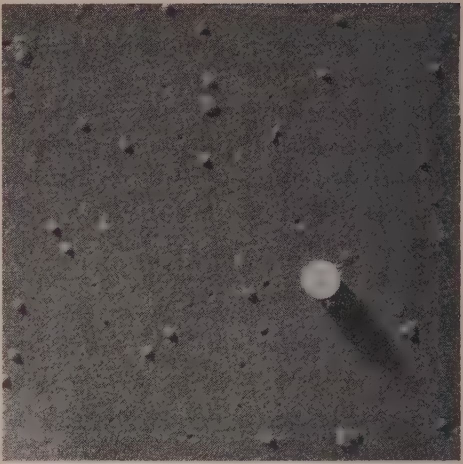

FIG. 1.

An electron micrograph of a purified preparation of Shope papilloma virus, shadowed with uranium. No attempt was made to remove all of the salt from the preparation. The diameter of the largest object shown, a polystyrene particle, is taken as 260 mμ.

Characterization of the virus preparations. The yields of virus obtained from different lots of warts ranged from about 0.1 to 0.3 mg of virus per gram of warts. The highly purified material induced warts on domestic rabbits to a dilution of 10^{-9} grams of virus. Examination of some of the virus preparations in the analytical ultracentrifuge and in the Tiselius electrophoresis apparatus by Dr. H. K. Schachman indicated the presence of a small amount of material differing in properties from the bulk of the substance(10). However, the bulk of the substance appears to coincide with the material shown by Beard and associates to represent the virus.

Several preparations of the virus were examined in the electron microscope. The resulting micrographs showed circular particles which appeared to possess considerable uniformity of size. One of these, kindly taken by Dr. R. C. Backus of this laboratory, is reproduced in Fig. 1. Included in the picture is a polystyrene particle, which coincides in size with other polystyrene particles not illustrated and hence on the basis of previous studies(11) can be taken as a spherical reference object with a diameter of about 260 mμ. From this, the diameters of the papilloma virus particles can be calculated to be about 73 mμ. However, from an inspection of the size and shape of the virus shadows as contrasted with that of the polystyrene particle, it is apparent that the virus particles are considerably flattened, as was also noted by Sharp and co-workers(12), and hence it is not possible to assign an accurate figure for the diameter of the unflattened particles. It is assumed from the uniformities of diameter and orientation that the particles in solution are essentially spherical. Further evidence suggesting this was kindly obtained for us in the following manner by Dr. R. C. Williams of this laboratory. An appropriate dilution of the preparation of virus illustrated in Fig. 1 was deposited upon a glass slide, allowed to dry, and then was washed twice with water to remove salt and dried again. The dried film was shadowed with palladium, and a replica was made in the customary manner. Several clusters of viral particles were found upon examination of the replica in the microscope. If the original viral particles were essentially spherical, it would be expected that the particles in a cluster, which are prevented from flattening to the same extent as isolated particles, would possess smaller diameters than those not in a cluster. Measurement across several particles in clusters yielded a figure of 62 mμ for the average diameter. This is significantly smaller than the diameters of isolated particles on the same micrographs. A still smaller diameter, 52 mμ, was found by a third technic in which the particles were sprayed and lyophilized on the microscope screen. The details of this method will be described later by Dr. Williams. Even with this last procedure there was some evidence for flattening of the particles, and hence the figure of 52 mμ should be taken only as a tentative value until more data, obtained with improved or other technics, can be brought to bear on the problem. It should be noted that the apparent flattening of the viral particles on electron microscope screens is not peculiar to papilloma virus, but is a phenomenon observed with all of the presumably spherical viruses thus far examined. Further investigations of the physical nature of the particles in papilloma virus preparations are being made and will be described later.

[†] In some of the preparations it was observed that appreciable amounts of clear gelatinous material sedimented with the brownish pigment in the angle centrifugation, and, furthermore, that much of this could be redissolved by mixing with more buffer; the ultracentrifuge pattern of this material was essentially the same as that observed for the supernatant fluid of the angle centrifugation, which is presumed to represent virus. Therefore, it seemed likely that the virus has a limited solubility under the above conditions and that this situation could be improved, as it was in one later preparation, by employing a buffer at a higher pH. 0.18 M sodium chloride—0.02 M sodium veronal at pH 7.4 was used for this purpose.

10. Schachman, H. K., in press.

TABLE I. Amino Acid Content of Preparations of Shope Papilloma Virus.

	Preparation	
	1	2
Amino acid	%	%
Alanine	—	3.1
Arginine	6.7	7.0
Aspartic acid	11.1	10.7
Cystine	—	6.4*
Glutamic acid	11.8	11.4
Glycine	—	3.1†
Histidine	1.9	1.8
Isoleucine	4.2	4.1
Leucine	7.0	7.5
Lysine	7.0	7.0
Methionine	2.2	2.1
Phenylalanine	4.5	4.6
Proline	—	5.5
Serine	—	4.0
Threonine	5.2	5.1
Tryptophane	—	1.0
Tyrosine	6.0	5.8
Valine	5.4	5.4

* A calculated value using Beard's figure for sulfur (4), subtracting methionine sulfur, and expressing the residual sulfur in terms of cystine. Preliminary colorimetric tests suggest that part of this sulfur is present as cysteine.

† No attempt was made to correct for the small amount of glycine possibly contributed from decomposition of nucleic acid constituents during hydrolysis of the virus.

Amino acid analyses—The dried preparations of papilloma virus were hydrolized and analyzed for amino acid content by the methods previously described(9). The results of these analyses are summarized in Table I. It should be noted that the two preparations analyzed were obtained in different years and were derived from warts obtained from many rabbits.

Discussion and summary. The results of the amino acid analyses indicated that at least 18 amino acids are represented in the

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protein of the Shope papilloma virus. None of these appear to be present in extraordinary amounts. In common with other viruses examined thus far(9,13,14), the protein of the papilloma virus has a preponderance of acidic amino acids over the basic ones, and hence differs from the proteins combined with nucleic acid in such substances as sperm. A summation of the amino acid residues and the nucleic acid in the papilloma virus leaves about 7% of the material unaccounted for. However, the nucleic acid figure taken from the data of Taylor *et al.*(7) is admittedly a crude estimate and, as judged from the phosphorus content of his preparations, is almost surely low. Further investigation of the nucleic acid content together with additional amino acid analyses may resolve the discrepancy, but at present the possible existence in papilloma virus preparations of material in addition to protein and nucleic acid cannot be excluded with the same degree of assurance as in the case of certain plant viruses. Finally, the amino acid values reported here must be considered subject to revision owing to the small number of analyses made.

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Received November 28, 1950. P.S.E.B M., 1950, v75.

Regular Diurnal Physiological Variation in Eosinophil Levels in Five Stocks of Mice.* (18365)

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Characteristic eosinophil levels for inbred, mature, male mice were defined in a previous study. They were found to be distinctive for some of the strains investigated(1). It was emphasized, however, that the determinations were made in the morning, at a time when the activity of the mice had presumably declined over a period of several hours from its high point around midnight(2). Furthermore, it was noted that the determinations made during the regular periods of maximal activity may differ from the "characteristic" level.‡

An additional study was therefore undertaken, designed to compare the midnight levels of circulating eosinophils in the tail blood of mice with the "characteristic" morning level.

Procedures and materials were comparable to those specified for the determination of the characteristic eosinophil morning level(1). The blood samples in which the night levels were determined had to be collected—for practical reasons—over a period of time from 9:00 P.M. to 1:00 A.M. Two independent series of determinations were made: both in midsummer, with an interval of a week between them.

The *results* are listed in Table I and indicate that under standardized conditions the numbers of circulating eosinophils show remarkable but regular variations. The significance

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* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and by research funds of the Graduate School of the University of Minnesota.

† With assistance of Arthur G. Litman, Victor M. Riddle and Paul C. Royce.

1. Halberg, F., Bittner, J. J. and M. B. Visscher, in press.

2. Bänder, A., *Z. f. d. ges. exp. Med.*, 1950, v115, 229.

‡ For the B_1 subline of the C_{57} Black strain the P value for the significance of the difference between the midnight level of 223 ± 23 cells per cu cm and the morning level of 1022 ± 44 cells per cu mm was smaller than 0.001.

TABLE I. Eosinophils Per cu mm of Tail Blood at 2 Different Periods of the Day in 5 Stocks of Inbred Mice.

Stock	N	Morning level Mean ± st. error	N	Midnight level Mean ± st. error
B_4 (Subline 4 of C_{57} black)	15	700 ± 59	7	369 ± 42
D_8 (Subline 8 of dilute brown)	14	297 ± 42	7	30 ± 10
Zb (Fostered line of C_3H)	14	198 ± 19	7	30 ± 6
Ax (Fostered line of A)	14	168 ± 15	7	55 ± 8
ZD_8F_1 Hybrids, Z° x D_8°)	14	130 ± 14	5	40 ± 15

TABLE II. Comparison of Differences Between Eosinophil Levels at 2 Periods of the Day in 5 Stocks of Inbred Mice.

Stock	Diff. ± S.E.*	t†	P_t‡
B_4	431 ± 92	4.68	<.001
D_8	223 ± 60	3.72	.002
Z_b	168 ± 27	6.23	<.001
Ax	113 ± 22	5.23	<.001
ZD_8	90 ± 26	3.48	.003

* Differences expressed as morning minus night means of eosinophils per cu mm tail blood (see Table I) with their standard errors.

† Assuming homogenous variance within samples.

‡ Probability that a difference as large as that observed would be expected by chance alone.

of difference between the values obtained at the respective periods of the day is presented in Table II. The values in column P_t, calculated by means of Student's *t* value, are indicative of significance below the one percent level.

This difference between the midnight low and the morning high level in all the strains investigated and in the absence of any exogenous stress suggests that eosinopenia may occur physiologically in mice which have not been artificially stressed. Since the mouse is a nocturnal animal the greatest activity, as noted above, occurs at night. The question may legitimately be raised whether normal activity constitutes a physiological stress capable of exciting the same structures and mechanisms as are activated by exogenous stresses(3-5).

It may be noted that a similar difference has been recently reported for the lipid content of the adrenal cortex in white mice killed in midsummer at two different times of the day(2). In animals killed at 9:00 A.M. the zona fasciculata was filled *in toto* with lipid. In mice killed at midnight the lipid did not extend beyond the outer third of the fasciculata.

Summary. In 4 inbred strains and in one hybrid group of mice a regular diurnal variation of large magnitude in the circulating eosinophil count has been demonstrated. On the average, in these strains, the midnight count was less than one third of the morning level.

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Received November 21, 1950. P.S.E.B.M., 1950, v75.

Transplantation of the Adrenal Gland in the Spleen of Rats. (18366)

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Previous communications(1,2) have reported the successful autotransplantation of the adrenal gland to the spleen of adrenalectomized animals. The present paper enhances the series previously reported(2), and includes data which suggest that reproduction is not inhibited when secretions from the adrenal

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2. Bernstein, D. E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 175.

gland pass through the portal circulation. In addition, this investigation describes the successful homotransplantation of adrenals of newborn rats into the spleen of adult castrated and non-castrated, adrenalectomized animals.

Method. (A) *Autotransplantation.* Forty-five immature Long-Evans rats were bilaterally adrenalectomized and one adrenal was placed in the spleen at the same operation. Postoperatively the animals were maintained on a regular diet and saline for 12 to 30 days, after which time the saline was replaced by tap water. The rats were surgically explored at various intervals from 30 to 200 days. At laparotomy, careful search was made for adhesions from the transplant or spleen that could circumvent the portal circulation. Two male and 5 female rats that had large adrenal transplants without adhesions, were later mated with each other and with normal animals.

(B) *Homotransplantation.* Twenty-five male and 31 female immature rats of the Long-Evans strain were bilaterally adrenalectomized, and at the same operation a single adrenal gland from newborn rats 1 to 4 days old, was transplanted to the spleen. In every case the donor and recipient of the homotransplant were of the same sex. Postoperatively the rats were given 2 injections of 5000 units each of penicillin and maintained on a regular diet and saline for 30 days, after which time the saline was replaced by tap water. The animals were weighed initially and at 45 days after operation, at which time autopsy was performed.

Results. (A) *Autotransplantation.* When the spleen was removed, 33 of the 45 animals showed viable transplants, which averaged 3 mm in diameter and on microscopic examination revealed adrenal cortical cells, with no medullary tissue present. Of the 12 animals in which no adrenal transplants were found, 5 died within 9 days after operation and were too autolysed for adequate necropsy. In 5 of the animals adrenal cortical tissue in the form of accessories was found, and in the remaining 2, neither transplants nor accessories were found. In no instance where successful transplantation occurred, were adrenal accessories or remnants discovered. When the rats with successful transplants were bred, it was found that all of the animals were sexually potent. The 5 females gave birth to normal litters which consisted of 3, 6, 8, 11 and 14 puppies respectively. Although 2 babies died within 10 days after birth, the mother that had 14 puppies nursed all, and the entire 14 survived to weaning with only one runt in the litter. Autopsy of the mothers revealed 3 to 5 mm adrenal transplants completely free of adhesions.

TABLE I. Tabulation of Results of Attempted Homotransplantation in Adrenalectomized Rats.

	Initial No. rats	Survivors after 45 days			
		Total No.	Transplants	Accessories	No adrenal tissue
Males	15	9	3	3	3
♂ castrates	10	6	4	2	0
♀	15	7	3	2	2
♀ castrates	16	12	4	8	0
Total No. rats	56	34	14	15	5

(B) *Homotransplantation.* At the time of autopsy 34 animals were still alive (Table I). In only 14 were viable transplants found. These averaged 3 mm in diameter and were composed of adult adrenal cortical cells with no medullary tissue present. In 15 of the remaining 20 animals, adrenal cortical accessories were found. In the other 5 rats neither transplants nor accessory adrenal tissue were discovered. In every case the animals that survived showed a good weight gain. The 22 rats that did not survive the experimental period were accounted for as follows: 19 died while still on saline, and 3 died within 5 days after being taken off saline. Autopsy was performed in 13 animals and revealed neither transplants nor accessory adrenal tissue; the remaining 9 animals were too autolysed for adequate examination.

Discussion. Butcher(1) attained 12 viable autotransplants from 14 experimental animals. No mention was made of adhesions from the transplant or spleen that could circumvent the portal circulation. Two of his rats from the above mentioned series were mated and bore litters of 7 and 8 puppies respectively, only 2 of which survived until weaning. The present report corroborates the finding that reproduction was not inhibited when the adrenal gland was transplanted to the spleen, but there is insufficient evidence to suppose that these females show inadequate lactation, as proposed by Butcher(1). The role of the adrenal gland in spermatogenesis, ovogenesis and pregnancy is still not clearly defined. However, several authors present evidence that at least for the rat, adrenalectomy inhibits the estrous cycle(3), and usually prevents pregnancy(4). The total lack of prevention of sexual activity in both sexes in the present investigation strongly suggests that the liver does not inhibit those factors in the adrenal gland responsible for adequate gonadal function in the rat.

In this report it is seen that autotransplants occurred in 35 of 45 animals. The number of surviving homotransplants obtained in the present investigation was 14 in 56 rats; 6 in 30 noncastrated and 8 in 26 castrated rats. Adrenal cortical accessories were found in 15 animals; 5 noncastrated and 10 castrated rats. The presence of accessory adrenal tissue would inhibit successful transplantation (2). Omitting the group in which accessory adrenal tissue was found, 14 of 41 animals had successful homotransplants. It is seen that takes in autotransplantation occur at least twice as frequently as in homotransplantation. From Table I, neither the sex, nor the presence of gonads significantly influenced the number of successful homotransplants. At the time of the author's previous report on autotransplantation(2), no definitive statement could be made about an increase in size of the transplants. However, since newborn adrenal glands are about 1 mm in diameter, it is obvious that surviving adrenal transplants to the spleen may increase in size (Fig. 1). Moreover, like autotransplants, the homotransplants maintained life and weight gain of the recipients.

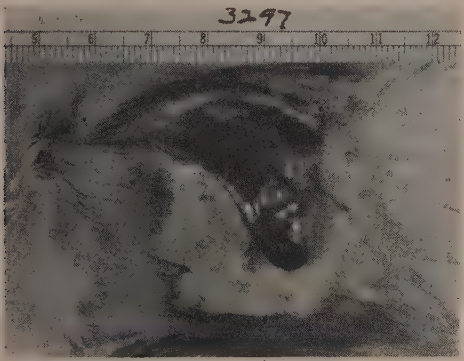

Fig. 1.
Homotransplant of newborn adrenal gland to spleen of an adult adrenalectomized rat, after 73 days.

Summary. 1. Thirty-three of 45 autotransplants of the adrenal gland to the spleen of adrenalectomized rats were successful. 2. Evidence is presented that the liver does not destroy those factors in the adrenal secretions responsible for the maintenance of spermatogenesis in the male, and pregnancy and parturition in the female rat. 3. Homotransplantation of the newborn adrenal gland to the spleen of adrenalectomized rats was successful in 14 of 56 animals, and is not significantly influenced by the sex of the recipient, or the presence or absence of gonads. 4. Homotransplants of the adrenal gland grew in castrated and noncastrated adrenalectomized rats, and maintained life and maturation of the recipients.

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Received October 23, 1950. P.S.E.B.M., 1950, v75.

Effects of Fat Meals and Heparin on Blood Plasma Composition as Shown by Paper Chromatography.* (18367)

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It has been shown in another study(1) that 6 to 9 hours after a large fat meal, and immediately after intravenous injections of a small amount of heparin the chylomicra and red blood cells have a tendency to aggregate. These changes were frequently attended by a decrease in the hematocrit, and by an increase in the sedimentation rate in non-lipemic plasma and a decrease in the sedimentation rate in lipemic plasma. The maximum aggregation of the chylomicra and red blood cells after a fat meal did not develop 3 hours after the fat meal when the chylomicra were most numerous, but 6 to 9 hours afterwards when the number of chylomicra were rapidly decreasing(1). Because of the possibility that this alteration of the suspension stability of the blood was due to factors which are protein in nature, preliminary studies of the total proteins, albumin, globulin and fibrinogen were carried out. No significant changes in these substances were found apart from a slight, but consistent reduction (about 0.25 g %) in the total proteins.‡

The recent application of the paper chromatographic technic to the study of plasma proteins(2-4) gives us a new tool which is both sensitive and easily applied to a study of blood plasma. With this method it has been possible to show changes in the proteins which correlate with the other changes in the blood described above(1). The application of paper chromatography to the study of proteins has been briefly reviewed in another paper(3).

Materials and methods. Two male and 4 female dogs weighing 10 to 21 kg were studied. A total of 23 experiments were performed in these 6 animals. Cream fat (relatively saturated) and a mixture of cod liver oil and linseed oil (relatively unsaturated) were tested; the test meals consisted of 4 g of fat/kg body weight. The maintenance diet of the dogs was beef heart and fox chow. Prior to the fat tests all dogs were fasted 18 hours unless otherwise indicated. All bloods were withdrawn from one of the leg veins and prevented from clotting by heparin-sodium salt (Connaught Laboratories). Occasionally isotonic ammonium and potassium oxalate were used as anticoagulants to provide controls on the heparin. 0.3 ml heparin (10 mg/ml) was injected intravenously. Observations were made of the number of chylomicra, the hematocrit, sedimentation rates, protein fractions (total protein, albumin, globulin and fibrinogen), and non-protein nitrogen. In the present experiments the hematocrit and sedimentation rates were used to indicate changes in the aggregation tendency of the red blood cells because these measurements are probably more dependable and less subjective than the changes observed in dark field illumination(1). The method of paper chromatography used in this study has been described in detail by Franklin and Quastel(3). Four sets of conditions were used to examine the plasma. One condition did not include the surface active agent, and the others used 0.02, and 0.03 ml Tween 81, and 0.03 Tween 85 per 0.5 ml of the test plasma.§

Results. The effect of intravenous administration of heparin in fasting dogs was

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* Supported by grants from the Multiple Sclerosis Society of Canada, and the National Cancer Institute of Canada.

† Canada Packers Research Fellow, McGill University.

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‡ It was also noted that the non-protein nitrogen fell 20% to 60% in 5 of the 6 experiments.

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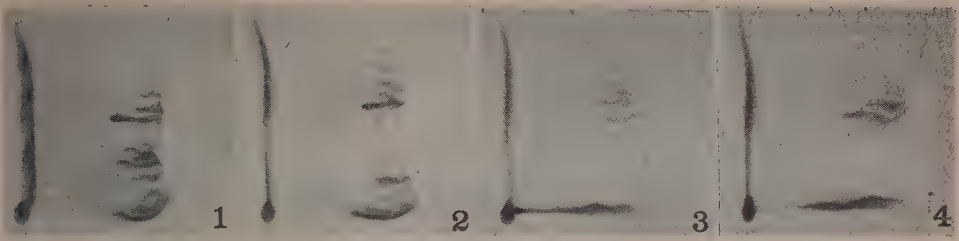

FIG. 1. Comparison between protein chromatograms of fasting dog plasma (No. 1), 6 hr after a high cream fat meal (No. 2) and 10 min. later after intravenous heparin (No. 3). No. 4 shows the protein pattern after intravenous heparin to the same dog after fasting.

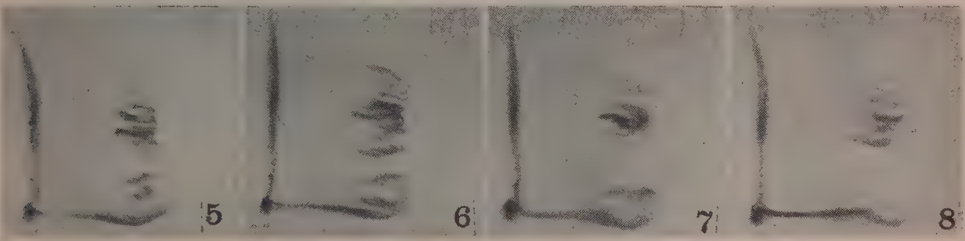

FIG. 2. Differences between fasting plasma (No. 5), 3 hr (No. 6), 6 hr (No. 7) and 9 hr (No. 8) after feeding of a high cream fat meal.

observed in 4 dogs, and 5 tests were made. In every instance a significant change in the protein pattern of the paper chromatograms was observed. The changes under one set of conditions in 1 dog are shown in Fig. 1 (Nos. 1 and 4). In these experiments the hematocrit fell from 2 to 4 points and the sedimentation rate usually increased(1). One additional experiment showed that the changed protein patterns observed in the plasma after intravenous heparin may return to normal in less than one hour. In this experiment the initial changes in the red blood cells and chylomicra were slight. This was frequently the case(1) when the dogs had been fasted for 24 to 36 hours.

Effect of a large cream fat meal with subsequent heparin administration was observed in 6 dogs on 8 occasions. Six hours after the fat meal, 7 plasmas showed definite paper chromatographic changes, presumably due to the fat absorption alone. In all 8 tests, heparin was injected 6 hours after the fat meal was given, and post-heparin samples of plasma were obtained 10 minutes later. All post-heparin bloods exhibited further alterations in the plasma protein patterns. The paper chromatograms for 1 such test are shown in Fig. 1 (Nos. 1, 2, 3). It is interesting to note that the changes produced by fat absorption alone appear to be intermediate between the fasting and post-heparin records in these experiments. The hematocrit showed a progressive decrease of 2 to 5 points during each of these experiments. The maximum change in the sedimentation rate was a reduction from 28 to 3 mm. A reduction of the chylomicron counts was also observed after the administration of heparin (1,5).

The effects of single large fat meals alone were observed 5 times with 2 dogs. Plasmas were taken at 0, 3, 6, and 9 hours after the fat meal. In 2 experiments, cream fat, and in 3 others a mixture of cod liver oil and linseed oil were used. The 3 tests with the mixture of very unsaturated fats all showed changes in the protein patterns at 3 hours, with maximum changes at 6 hours. The

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§ We are indebted to the Atlas Powder Co., Wilmington, Del. (per G. F. Sterne & Sons, Brantford, Ont.) for samples of Tween 81 (Polyoxyethylene sorbitan monooleate) and Tween 85 (Polyoxyethylene sorbitan trioleate).

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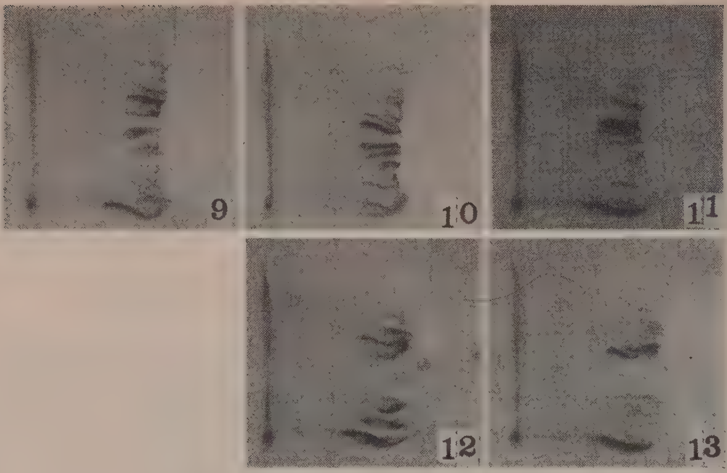

FIG. 3.
Effect of high speed centrifugation on the plasma of dogs which have been fed a high fat meal. No. 9 is the chromatogram of a fasting plasma, No. 10 of the clear fraction of the plasma of a 3 hr lipemia, No. 11 is the same at 6 hr, No. 12 is the lipemic portion at 3 hr and No. 13 is the same at 6 hr.

chromatograms returned to very nearly normal at 9 hours. With the relatively saturated fat one dog exhibited no change; the other exhibited marked changes at 6 and particularly at 9 hours after the fat meal (Fig. 2; Nos. 5, 6, 7, 8). The dog which exhibited marked changes in his plasma proteins exhibited also a drop in the hematocrit (from 50% to 44%) and an increase in sedimentation rate (from 3 mm to 9 mm) at 9 hours, at a time when the chylomicron count had returned about to normal. In the tests with unsaturated fat less marked changes were observed in the hematocrit and sedimentation rate.

The effects of separation of most of the chylomicrons from plasmas by centrifugation at about 13,000 r.p.m. (R.C.F. 20,000 x gravity) were observed in 2 experiments. This gave 2 plasma samples of equal volume, one clear and the other very cloudy, for protein analysis. In Fig. 3 it can be seen that the cloudy portion of plasma (Nos. 12, 13) exhibits definite and similar changes at both 3 and 6 hours, and that lesser changes are to be observed in the clear plasma at 6 hours (No. 11) and none at 3 hours (No. 10).

The effects of intravenous administration of Tween 81 were determined twice in the same dog. On both occasions 5 ml of Tween 81, diluted to 20 ml with distilled water, was injected; once with the dog fasting (Fig. 4; Nos. 14, 17) and subsequently 4 hours after a fat meal (Fig. 4; Nos. 14, 15, 16). During the first test the dog became listless, very weak, and collapsed a few minutes after injection of the Tween, but did not lose consciousness. During the next test, 2 weeks later, the dog was physically unaffected by the Tween injection, which was given at the presumed height of lipemia. In both tests the injection of Tween 81 altered the chromatograms remarkably. Due to the fact that the clustering of chylomicra was much greater in plasma than whole blood(1) red blood cells from 2 normal humans were washed with a volume of normal saline equal to the removed plasma, and chromatograms of this saline were made. A trace of a protein fraction was observed in each experiment, which was believed on the basis of later evidence¶ to be an albumin.

Discussion. The change in the protein pattern in the chromatograms described in this paper is probably not due to an overall reduction in the plasma proteins, since these

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¶ Unpublished data (Franklin and Quastel).

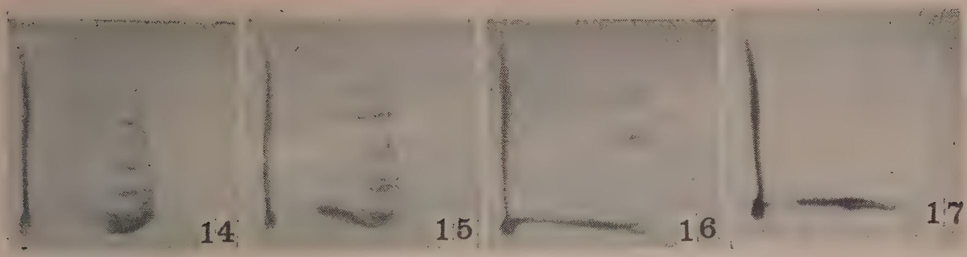

FIG. 4.

No. 14 is the protein chromatogram of a fasting dog plasma. No. 15 is a 6 hr lipemia, No. 16 is 10 min. after the intravenous injection of Tween 81 given after 6 hr of lipemia. No. 17 shows the effect of intravenous Tween 81 on a fasting dog.

NOTE: In all illustrations, 0.03 ml Tween 81 is mixed *in vitro* with 0.5 ml heparinized plasma, and 0.02 ml 0.3% hemin added. 0.02 ml aliquots are applied to the paper.

changed so little. More likely, specific fractions were affected selectively and in a way that altered the protein complex, and consequently its distribution in the chromatograms. The fact that the changes in the chromatograms may be accompanied by increased clumping of the red blood cells and chylomicra, by a decreased hematocrit, and by changes in the sedimentation rate raises the question as to whether the protein complexes in question are associated with the maintenance of the stability condition of the blood. The protein changes were sometimes attended by little or no evidence of physical change in the blood, such as no significant change in the hematocrit and sedimentation rate, indicating that there is a critical level for these protein complexes beyond which the emulsification of the blood elements becomes unstable. The absence of increases in the globulin and fibrinogen is of interest since these factors are increased under certain circumstances in which the sedimentation rate is also increased (6).

The similarity of the changes in the protein patterns, and in the physical characteristics of the blood, both after a high fat meal and after intravenous heparin, and the apparent cumulative nature of these changes suggest that their mechanisms are similar. It has already been suggested (1) that the chylomicra which appear in the blood stream after a fat meal may, by competing with the red blood cells, produce a deficiency in the so-called emulsification factors. This deficiency may last no longer than 3 to 6 hours as indicated by changes in the physical state of the blood (1), and in the protein pattern. The action of heparin is not clear, but it may be that this substance, either directly or indirectly, causes changes in the plasma by surface active forces resulting in alterations in the protein patterns. The duration of this change may be less than one hour. Anderson and Fawcett (7) have shown that the surface tension of plasma decreases after injection of heparin effective in clearing the lipemia. These authors ascribe this to the formation of a surface active heparin-phospholipid complex.

The paper chromatogram observations on lipemic plasma, subjected to very fast centrifugation, indicate that the changes in the protein patterns are not due to the presence of the chylomicra alone. It is also evident that a very marked lipemia produces additional changes in the paper chromatogram resulting in protein patterns similar to those seen after intravenous heparin. As further evidence that the presence of chylomicra is not the sole cause of the protein changes, it is to be noted that the maximum changes in the proteins and physical characteristics of the blood were observed in the cream fat test in one dog (Fig. 2) 9 hours after the fat meal, when the number of chylomicra had returned nearly to normal; at 3 hours when the lipemia was maximum no such changes in the protein

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7. Anderson, N. G., and Fawcett, B., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 768.

patterns were observed.

The evidence would indicate that the administration of heparin, as well as a high fat meal, modifies the surface active substances in the plasma with the result that a marked change occurs in the protein pattern seen in the paper chromatograms. This is noted also in the effects due to administration of a surface active agent such as Tween 81. It has been shown recently(8) that heparin inhibits the action of hyaluronidase, possibly by competition with hyaluronic acid for the enzyme. Conceivably the change in the protein chromatogram of a plasma after heparin administration is due to some similar association with a protein fraction.

Summary. 1. Changes in the plasma protein patterns of dogs after high fat meals, and after the intravenous administration of heparin or Tween 81, have been demonstrated, using the paper chromatographic technic of Franklin and Quastel. 2. Concurrent reductions in the hematocrit, and changes in the sedimentation rate were observed after these procedures. The chylomicron counts were decreased after intravenous heparin. 3. The evidence indicates that the surface active substances present in the blood are modified after the above administrations, thus affecting the protein patterns of the paper chromatograms.

We wish to acknowledge the technical assistance of Miss Aagot Grimsgaard.

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Received Novermber 8, 1950. P.S.E.B.M., 1950, v75.

Role of Cortisone in Regulation of Inflammation.*† (18368)

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It is well established that certain generalized reactions such as lymphocytolysis(1), lymphocytopenia(2) and eosinopenia(3,4), which occur following the application of noxious stimuli, are mediated by the secretion of the adrenal cortex. However, the role of the secretion of the adrenal cortex in localized host reactions elicited by topically applied stimuli has not yet been evaluated. Outstanding among these local reactions is the process of inflammation. The term "inflammation" is used here to denote only the local reactions that occur when an injurious agent acts topically upon tissues. It is the object of this study to secure information on the influence of the secretions of the adrenal cortex upon each of the various component events composing the inflammatory response. A stimulating agent was chosen which was not only known from prior studies to produce typical inflammatory alterations, but which has also been implicated in the etiology of connective tissue disease. To eliminate the influence of endogenous hormone secretion, which is difficult to estimate, adrenalectomized animals were used. The involvement of secretion of the adrenal cortex was then studied by comparing the inflammatory reaction of untreated adrenalectomized animals with that of adrenalectomized animals treated with a standard dose of cortisone acetate (Cortone acetate, Merck & Co.).

Materials and methods. Approximately 150 CBA mice 16 weeks of age, of both sexes,

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* This investigattion was supported by a grant from the Life Insurance Research Foundation.

† The authors wish to thank Dr. S. Loewe of the Department of Pharmacology, University of Utah College of Medicine, for his valuable aid in preparation of this manuscript, and Miss K. Seymour for technical aid.

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2. Dougherty, T. F., and White, A., *J. Lab. and Clin. Med.*, 1947, v32, 584.
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4. Thorn, G. W., *The Diagnosis and Treatment of Adrenal Insufficiency*, Charles C. Thomas, 1940.

were used in these experiments. All animals were kept at a constant external temperature and were fed *ad libitum* on dog chow (Purina), supplemented with calf meal (Larro), vitamins and water. The animals were sensitized with 0.7 cc and 0.5 cc of horse serum given intraperitoneally in an interval of 2 days. The local inflammation was then produced 19 days after the second sensitizing dose by topical subcutaneous injection of a challenging antigen dose into the subcutaneous tissue of the abdomen. All animals received a uniform challenging dose, namely 0.0005 cc horse serum per 20 g mouse given in a total volume of 0.25 cc saline. This dose was selected because it had previously been established as the anaphylactic LD_{50} in adrenalectomized animals(5), when injected intravenously. The volume was chosen to insure sufficient spread of the antigen to produce an area of response adequate for the subsequent biopsy. The mice were adrenalectomized in the customary fashion by the bilateral posterior approach. In all experiments, the challenging dose was injected 3 to 4 hours after adrenalectomy. The area injected was marked with wax pencil for exact identification of the site of topical treatment. The adrenalectomized animals were divided into 2 groups. To Group 1, 1 mg of cortisone acetate per 20 g mouse was given by the intraperitoneal route 2 hours after adrenalectomy and 2 hours before the administration of the challenging dose of horse serum. The second group received only the antigen dose at the same time interval after adrenalectomy. Five groups of control animals were used in these experiments: (1) non-sensitized intact animals. (2) sensitized intact animals. (3) sensitized adrenalectomized animals and (4) sensitized adrenalectomized animals given 1 mg of cortisone acetate by intraperitoneal injection 2 hours after adrenalectomy. These 4 groups received no local serum injection. The fifth control group was composed of intact sensitized animals receiving the challenging antigen dose 19 days after the second sensitizing dose. This control group was introduced in order to study the inflammatory response in sensitized non-adrenalectomized animals. In all instances at least 7 animals were studied at each of the time intervals at which subcutaneous spreads were taken, with the exception of the control Groups 2 and 5, which consisted of 5 animals.

The methods for studying the inflammatory reaction were essentially those described in detail previously(6). At varied intervals following administration of the antigen, the skin of the challenged area was opened and the subcutaneous connective tissue excised in thin sheets for the study of the local inflammatory response. Such spreads were taken 2, 4, 6, 8, 10, 12, and 24 hours after the injection of the antigen dose from all challenged animals, 6 hours after removal of the adrenals from the adrenalectomized, and 4 hours after cortisone from the intact controls. The excised sheets of areolar tissue were spread thinly on slides, air-dried and stained with May-Grünwald Giemsa. Six or 8 spreads were prepared from the area of inflammation and at the same time control spreads were taken from other areas far removed from the injected zone. Moreover, loose connective tissue spreads were prepared simultaneously from the site of incision for adrenalectomy. In those groups in which no topical inflammatory phenomenon was arranged, the spreads were taken from a comparable subcutaneous site. In each spread, in addition to an extensive qualitative cytological study, differential cell counts were made, ascertaining the percentage distribution of fibroblasts, polymorphonuclear leucocytes, macrophages, lymphocytes and eosinophils. A differential count of 800 cells was performed on each set of spreads originating from the site of horse serum injection.

Results. Comparison of the control groups revealed that sensitization alone or combined with adrenalectomy did not produce any marked alteration in either morphology or number of the cells of subcutaneous connective tissue. The eosinophils in areolar tissue were markedly decreased in all sensitized animals. The same holds true for sensitized intraperitoneally cortisone-treated controls.

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TABLE I. Changes in Percentage Distribution of Cells in Allergic Inflammation Produced By Locally Applied Horse Serum.

Group	Time,[*] hrs.	Fibroblasts Mean[†] % S.E.	PMNs Mean % S.E.	Macrophages Mean % S.E.	Lymphocytes Mean % S.E.	Eosinophils Mean % S.E.
1. Non-sensitized intact controls		88.3 ± 1.26	0	.7 ± .38	2.2 ± .33	8.2 ± 1.12
2. Sensitized intact controls. + .0005 cc H.S. s.c.	6	58.5 ± 5.70	31.2 ± 5.82	3.0 ± 1.00	2.3 ± .73	4.5 ± 1.00
3. Sensitized adrenect local injection of .0005 cc H.S. s.c.	2	69.7 ± 4.98	20.1 ± 5.88	1.6 ± .51	4.9 ± .71	3.1 ± .79
	4	40.4 ± 5.43	49.9 ± 5.46	1.6 ± .34	3.9 ± .52	3.5 ± .91
	8	29.5 ± 3.35	61.5 ± 4.20	2.5 ± .70	2.3 ± .44	3.5 ± 1.13
	12	30.8 ± 4.61	49.8 ± 4.62	11.9 ± 1.88	1.5 ± .40	5.3 ± .90
	24	29.7 ± 4.09	30.3 ± 2.03	30.9 ± 3.08	2.6 ± .47	6.0 ± 1.03
4. Sensitized adrenect + 1 mg Cortisone i.p. + .0005 cc H.S. s.c.	2	79.2 ± 4.98	6.9 ± 3.97	4.1 ± .85	5.2 ± .71	4.4 ± .92
	4	75.7 ± 4.40	17.2 ± 4.47	2.1 ± .41	3.3 ± .53	1.3 ± .33
	6	69.0 ± 4.73	18.5 ± 5.68	1.2 ± .37	4.0 ± .91	10.6 ± 1.86
	8	77.4 ± 3.55	17.1 ± 3.63	.9 ± .28	1.9 ± .61	2.7 ± .52
	12	79.6 ± 7.59	13.9 ± 3.39	1.4 ± .54	1.4 ± .52	2.9 ± .67
	24	80.9 ± 2.42	11.0 ± 2.83	1.5 ± .46	2.0 ± .30	4.7 ± 1.00

[*] After local application of H.S.

[†] All means and standard errors represent values obtained on 10 animals, with exception of Group 2 in which 5 animals were used.

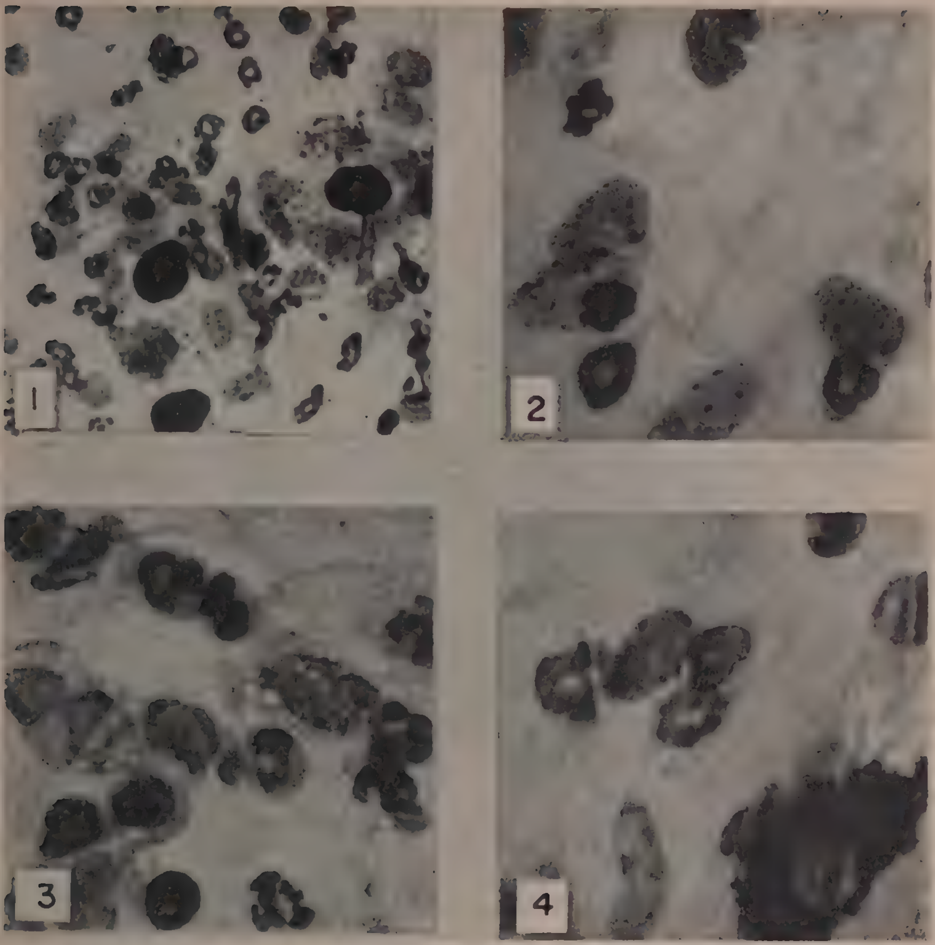
The local inflammatory response of sensitized animals to the challenging antigen injection was qualitatively the same in intact (Table I, Group 2) and in adrenalectomized animals (Table I, Group 3). Within the first 2 hours following administration of the challenging dose of antigen, the connective tissue at the site of injection exhibited localized edema. The fibroblasts underwent early degenerative changes such as plasmolysis, swelling of nuclei and, in many instances, actually karyorrhexis (Plate 1, Fig. 1). The inflammatory response was quite different when cortisone was administered 2 hours before the challenging dose (Table I, Group 4). Whereas the connective tissue became edematous, the destruction of fibroblasts and the invasion of polymorphonuclear leucocytes and macrophages were much less extensive.

The above described qualitative findings are supplemented by the quantitative data obtained from the differential cell counts in the inflamed areas.

The abnormal percentage distribution characteristic of the local inflammatory response exhibits the same trend in the intact group (Table I, Group 2), and in adrenalectomized animals (Table I, Group 3); the shifts in cell count after 6 hours appear more pronounced in the adrenalectomized animals, particularly the decrease in fibroblasts and the invasion of macrophages. In the cortisone-treated animals, the inflammatory response is quite different, as illustrated by a comparison of the data for percentage cell distribution in the areas of inflammation of cortisone-treated (Table I, Group 4), with those of non-cortisone-treated animals (Table I, Group 3). In the cortisone-treated group (Table I, Group 4) the inflammatory response was not completely suppressed but it was greatly diminished and shortened.

The traumatically induced inflammation in the adrenalectomy wounds paralleled in both groups the allergic inflammation in the serum-challenged area, *i.e.* exhibited a similar diminution by systemic cortisone treatment.

Comment. In the present study, a local antigen-antibody reaction was used as the method of producing acute focal inflammation. The local inflammatory phenomena here described demonstrate that the cytological changes and their temporal characteristics are similar in such allergen-produced inflammation to those inflammations produced by various other inflammatory stimuli, *e.g.* traumatic, chemical or bacterial(6,7). Technically, the procedure employed focuses the



All Preparations were Air-dried and Stained with May Brünwald Giemsa Solution.

FIG. 1. PMN-response in loose connective tissue 4 hr after local horse serum injection to sensitized adrenalectomized animals. Fibroblasts present in the injection area show cytoplasmic and nuclear destruction. ×540.

FIG. 2. Connective tissue of cortisone (1 mg i.p.) treated animals 4 hr after local horse serum injection to sensitized adrenalectomized mice. Few PMN's and eosinophils present. Few injured fibroblasts. ×760.

FIG. 3. Macrophage response in untreated animals 24 hr after local horse serum injection to sensitized adrenalectomized mice. Thirty per cent of cells present are macrophages, approximately 30% PMN's and another 30% fibroblasts. ×760.

FIG. 4. Connective tissue of cortisone treated animals 24 hr after local horse serum injection to sensitized adrenalectomized mice. Note absence of macrophages and slight increased number of locally developing eosinophils. Mast cell in the lower right corner. ×760.

observation to the study of the primary arena of those exacerbations of physiologically fluctuating processes, called inflammation, and to their major performers the cellular elements of connective tissue. Regarding the stimulus employed, the method is attractive because the inflammatory process is produced by allergy, which more and more emerges as an

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7. Menkin, V., Dynamics of Inflammation, Macmillan Co., 1940.

important factor in the production of connective tissue disease(8,9). Furthermore the method allows the detailed study of the changes in cellular morphology, the fluctuations in cell population and the alterations in tissue structure in the sequence in which they characterize the destructive, the defensive and the reparative stage of inflammation.

Adrenal cortical secretions unquestionably exert a marked antiphlogistic action. This is indicated by the results of adrenalectomy, which noticeably increases the inflammatory response and by cortisone treatment which profoundly depresses the cellular changes of inflammation.

This inhibitory effect of cortisone involves both the alterative process of fibroblast damage and the invasion of non-autochthonous cells. The influx of polymorphonuclears is markedly diminished by the adrenal hormone. Analysis of the data in Table I would seem to indicate the cortisone treatment does not influence the invasion of lymphocytes. This, however, is not the case. Since the lymphocytes entering the field of inflammation in non-cortisone treated adrenalectomized animals transform rapidly to macrophages, a low value for lymphocytes is maintained at the various intervals studied. The absence of a macrophage response to the phlogogenic stimulus in cortisone-treated animals could be a result of the capacity of this hormone to minimize lymphocyte invasion and thereby eliminate the cells which are known to be the precursors of macrophages in the inflamed area(6,10,11). It is possible that by this mechanism, administration of large amounts of cortisone or hypersecretion of adrenal cortical hormones can diminish the resistance of the organism to many noxious stimuli.

Adrenal cortical hormones could exert their antiphlogistic effect upon allergic inflammation through one or two major mechanisms. They could interfere either with the antigen-antibody reaction itself or with its sequels. Regarding the former mechanism, cortisone may increase the amount of circulating antibody, which would bind more antigen and leave less free for reaction with cellular antibody. It is possible also that cortisone might decrease the amount of cellular antibody or prevent its union with antigen. The literature is completely contradictory concerning the influence of adrenal cortical hormones on the level of circulating antibody. Some claims exist that adrenal cortical secretions enhance circulating antibody titer(12-15). On the other hand it has recently been suggested that adrenal cortical hypersecretion diminishes circulating antibody(16,17). The controversial reports on the producibility of an anamnestic response(13,18-20) by these hormones further increases the contradictory status. That cortisone does not prevent the *in vitro* Schultz Dale reaction(21) nor the tissue alterations of anaphylactic shock *in vivo*(5) indicates that the antiphlogistic effect of cortisone is not due to interference with antigen-antibody union.

It has been suggested(5) that cortisone may inhibit allergic inflammation not by some mechanism specifically related to the antigen-antibody union but rather through its general antiphlogistic action. Evidence which supports this view is that acute inflammations

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produced by trauma are inhibited as well as those originating from chemical stimuli(22). There is much evidence emphasizing that the substances released by the antigen-antibody reaction elicit the responses of the effector organs, tissues and cells. The influence of cortisone upon the release, transfer, distribution or action of these mediator substances will be of primary importance for future investigations designed to ascertain the mechanism of the antiphlogistic action of this hormone.

Summary. By investigation of experimentally induced allergic and traumatic inflammation in (a) adrenalectomized and (b) adrenalectomized and cortisone-treated animals, the role of the adrenals and of exogenous cortical hormone in the regulation of the cellular changes of the inflammatory process was evaluated. Adrenal cortical secretions and exogenous cortisone were demonstrated to inhibit allergic inflammation through an antiphlogistic action *sui generis,* rather than by interfering with the antigen-antibody union. It is suggested that this antiphlogistic action of cortisone accounts for the capacity of this hormone to minimize the consequences of a wide variety of unrelated inflammation producing stimuli.

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Received October 23, 1950. P.S.E.B.M., 1950, v75.

A Study of Elastic Arteries in Irradiated Mice of Different Ages.* (18369)

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In a recent paper(1), aging changes in the tunica media of the aorta of animals from newborn through old age were described. The present study is concerned with the effects of x-radiation on the media of elastic arteries of mice of different age groups. A review of previous work on the effects of irradiation on blood vessels has been presented by Rhoades (2). She reported that "the most constant and obvious effects of irradiation . . . were changes in collagen" and that these changes could be "considered characteristic of the treatment." Her discussion was concerned primarily with the tunica adventitia; however, since that portion of the arterial wall is poorly developed in the mouse, a study of the well developed media was considered more feasible.

Methods. Fifteen mice, 20 to 30 days of age, 15 mice, 80 to 100 days, and 15 as old as could be obtained from the Roscoe B. Jackson Memorial Laboratory were used in this investigation. X-ray of 250 kv filtered with 2 mm of copper and 1 mm of aluminum was used for total body external exposure. The animals were held in a plastic wire cage(3) and received single treatments of 410 r. The intensity of x-radiation was 31 r per minute. According to Murray(4), the dose lethal to 50% of the mice within 30 days has been determined by Hagen, Simmons and Sacher to be about 500 r. Mice from each age group were killed at 2,4,8,12,22,28,36,48,72,84,96, 117 hours and 6,7,8 days after x-radiation. The aortae of 58 mice studied for aging changes in the media from birth through 760 days of age were used as controls(1).

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* This work was supported by a grant from the United States Public Health Service. The mice were irradiated through the courtesy of the Westfield State Sanatorium.

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The region of the ventral mediastinum which included the thymus and sections of elastic arteries was fixed in ice-cold Rossman's fluid, embedded in paraffin and sectioned at 5 μ. Sections were treated with 0.5% aqueous toluidin blue, periodic acid-leucofuchsin(5), the Feulgen reagent for thymonucleic acid, resorcin fuchsin, orcein, hematoxylin and eosin, and Mallory's connective tissue stain. Sections impregnated with silver (Foot's modification of the Bielschowsky technique) were either unstained or stained in orcein or Mallory's connective tissue stain. Sections of elastic arteries were also mordanted in ferric chloride or copper sulfate and stained in Mallory's unoxidized hematoxylin for the demonstration of ground substance. This technic was not reliable and the observations were therefore not significant.

Results. The effects of x-radiation on the elastic membranes of the arteries were studied in sections stained in orcein, resorcin fuchsin, and periodic acid-leucofuchsin. In some arteries of animals in the 20 to 30 days old group, the deeply colored (in elastic stains), predominantly smooth membranes in certain regions of the wall were interrupted by ragged and frayed areas. This raggedness was not apparent in control animals of the same age group. Membranes of arteries from experimental and control animals exhibited a greyish ivory color after the periodic acid-leucofuchsin technic. In certain areas the membranes appeared to be Schiff positive due to heavy investment with reticular fibers. Membranes in sections digested by saliva were more intensely colored by the Schiff reagent. A positive reaction was obtained in both experimental and control elastic membranes tested by the Feulgen method for thymonucleic acid. Since the lamellae appeared colored in sections in which the previous hydrolysis in HCl had been omitted, the rosy purple was not due to the reaction of the Schiff reagent with thymonucleic acid. As in the aortae of the control animals, the elastic lamellae of all age groups were uncolored by toluidin blue. In contrast to the youngest group of animals, the elastic membranes in the arteries of the 80 to 100 days old irradiated mice were predominantly ragged with some smooth areas. Splitting of the membranes was observed in a few arteries. After the elastic stains, the periodic acid-leucofuchsin and the Feulgen technics, the membranes were paler than those of the youngest group. This decrease in depth of color was similar to that seen in the aortae of the control animals. The membranes of the old irradiated mice were decidedly ragged and frayed, and paler in orcein and the other dyes than in either of the two younger groups. The elastic lamellae were colorless in sections treated with periodic acid-leucofuchsin. In most arteries the membranes were a pale delicate purple after the Feulgen technic, but in a few instances they were colored by the counterstain, fast green. Since the structure and tinctorial reactions were the same in both control and experimental vessels, no effects of x-radiation were observed on the membranes of the old animals.

Interlamellar fibers and reticular sheaths of the membranes were studied in all preparations. The results obtained with silver impregnation were the same as those in the periodic acid-leucofuchsin technic; those fibers which precipitated silver recolored the Schiff reagent. The walls of the irradiated arteries of the two younger groups, because of more interlamellar elements, seemed denser than those of the controls. In the group 20 to 30 days old, the reticular sheaths of the elastic membranes varied in thickness from ¼ to ½ the width of the lamellae. Interlamellar fibers were generally fine and few in number. The thickness of the reticular sheaths was not measured but, as in the controls, appeared thicker under the inner elastic membrane. The interlamellar fibers were heavier and the sheaths varied from ½ to the whole width of the membranes in the group 80 to 100 days old. In some of these vessels there were regions in which the sheaths and interlamellar fibers were so dense that they resembled older arteries. The width of the fibrous sheath in the oldest group of experimental and control animals ranged from ½ to greater than the width of the membranes. After silver impregnation or after staining in periodic acid-leucofuchsin, the whole wall ap-

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peared more deeply colored due to the increased density of the interlamellar fibers as well as the heavier sheaths.

Fine and sometimes coarse elastic interlamellar fibers were observed in the 20 to 30 days old arteries stained in orcein and resorcin fuchsin. In contrast such fibers were not prominent until about 70 days of age in the control series. There was no conspicuous difference in density of these fibers in the arteries of the control and irradiated mice of the two older groups.

The effect of x-radiation on the ground substance was studied mainly in sections stained in toluidin blue. Metachromatic staining was found in focal areas of the walls of the 20 to 30 days old arteries where the colorless membranes were outlined by a narrow rose purple line and a fine mesh of the same color was seen between the lamellae. Metachromasia was stronger than in the aortae of control mice even though it was not found in all arteries and varied in intensity and extent. In general the strongest reactions were seen under the inner elastic membrane and/or in close association with the muscle cells. The cell membranes of the muscle cells in the arteries of all age groups in these preparations were so delicate that it was often impossible to distinguish them from the ground substance; however, where cell membranes were recognized, metachromatic granules outlining vacuoles were seen within some muscle cells as well as in the ground substance. In the arteries of the 80 to 100 days old mice, the metachromatic reaction was more intense and the localized regions more extensive than in the younger vessels and than in the controls. A more generalized and paler tint was seen throughout the walls. First noticeable at 8 hours after irradiation, the coloring was strong at 12 hours, more intense in most of the succeeding hours, and present in all the remaining arteries of the series. Metachromatic staining of the ground substance was observed in variable degree in arteries of the whole series of old animals; the color reactions were sometimes pale, and sometimes intense. As in the non-irradiated mice, the metachromasia was more striking in old arteries than it was in the younger vessels. Although the number of animals used was too few to study individual variations, these observations have indicated that the effect of x-radiation on the walls of elastic arteries in young animals was to produce premature aging changes. Evidences of these changes in mice 20 to 30 and 80 to 100 days old were the raggedness and fraying of the membranes, the increased amount of ground substance and the greater numbers of interlamellar fibers. X-radiation produced no detectable changes in elastic arteries of old mice.

Within each age group, the number of hours elapsing between irradiation and the sacrifice of the animal made no observable difference in the reactions of the arterial wall to most stains. With toluidin blue, however, there were indications that the metachromatic ground substance might be increased as the time interval between irradiation and sacrifice of the animal was lengthened. The small number of animals employed and individual variations may have masked any other patterns which might have been present.

Summary. Changes comparable to those of physiological aging were induced by x-radiation in the tunica media of elastic arteries of mice of different ages. In the elastic arteries of 20 to 30 days and 80 to 100 days old irradiated mice there were increased amounts of ground substance, increased numbers of interlamellar fibers, and premature raggedness and fraying of the elastic membranes. No changes attributable to irradiation were seen in the arteries of the old mice.

Received November 20, 1950. P.S.E.B.M., 1950, v75.

Studies on Proteolytic, Fibrinolytic, Antitryptic and Antifibrinolytic Activities in the Blood of Rats. (18370)

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The possible importance of the equilibrium between plasmin* ("fibrinolysin") and its inhibition in relation to clot lysis, blood coagulation, protein breakdown, and the body's response to various stimuli has been recognized only recently(1,2). It has been established that various types of stresses will cause an increased breakdown of protein. The nature of the mechanism of this physiological process is at present little understood.

This paper describes certain experiments, designed to contribute to an understanding of the sequences of increased protein catabolism observed under various conditions of stress.

Experiments and results. Male Wistar rats, weighing 215 to 330 g and fed on Purina dog chow checkers, were used. The animals were fasted and water was withheld for 24 hours before the experiment. The procedure for shock production was the same as previously described(3,4). Elastic rubber bands (Eberhard Faber No. 30) were applied to both hind limbs for 4½ hours and the blood drawn by heart-puncture 2 to 11½ hours after tourniquet release. It was found that the conditions described yielded a 24 hour mortality of 96%.

A. *Proteolytic and antitryptic activity of blood of rats in "tourniquet shock."* The proteolytic activity (pH 7.3) of the serum of normal control rats, determined according to Downie and Cliffton(5), was found to be practically zero, and no increase could be demonstrated in the serum of rats in "tourniquet shock" (total of 47 animals; blood drawn 2 to 16 hours after release of the tourniquets). Chloroform activation(1) did not reveal either any differences in the proteolytic activity of the serum of normal and shocked rats. The activity after chloroform treatment (25°, 16 hr) corresponded to about 8γ of trypsin per ml of serum in both groups (total of 83 animals). The antitryptic activity, determined essentially according to Tallan, Cliffton and Downie (6), was found to be slightly, but not significantly, lower than in the serum of normal rats (total of 84 animals).

There was the possibility that the tests employed in measuring proteolytic and antitryptic activity were not sufficiently sensitive to detect small changes in the enzymatic activity of serum. It was, therefore, investigated whether the measurement of the fibrinolytic and antifibrinolytic activity might be a more sensitive, possibly significant indicator during conditions of stress.

B. *Action of various proteolytic enzymes on the degree of clotting ability and total hydrolysis of fibrinogen.* No data seem to be available comparing the action of proteolytic enzymes on the clotting ability of fibrinogen and the hydrolysis to non-protein compounds. Fibrinogen, therefore, was incubated with the enzyme preparations listed in Table IB. The decrease in the clotting ability of fibrinogen was followed simultaneously with an increase in the amount of non-protein tyrosine. The extent of the loss of the clotting ability was determined by measuring up to what dilution the fibrin clot was detectable, or what con-

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* This term is used throughout, following the suggestion made in the Transactions of the First Conference "Blood Clotting and Allied Problems," Josiah Macy, Jr. Foundation. p38, New York City, 1948.

1. MacFarlane, R. G., and Biggs, R., *Blood,* 1948, v3, 1167.

2. Mole, R. H., *J. Path. Bact.,* 1948, v60, 413.

3. Hamolsky, M. W., Gierlach, Z. S., and Jensen, H., *Am. J. Physiol.,* in press.

4. Gray, J. L., Botkin, A. L., Moulden, E. J., and Jensen, H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 189.

5. Downie, G. R., and Cliffton, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 138.

6. Tallan, H. H., Cliffton, E. E., and Downie, G. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 667.

TABLE I. Effect of Various Proteolytic Enzymes On Clotting Ability and Total Hydrolysis, In % of Total Fibrinogen.

A. *Fibrinogen conc. and clotting time*
All sol. in veronal pH 7.35.
Fibrinogen sol. = 20 mg fibrinogen, containing 40-50% citrate, in 6 ml veronal.
Clotting time = 1 ml of diluted fibrinogen + 0.5 ml of 0.5% hemostatic globulin.

mg Fibrinogen per 1.5 ml of final reaction mixture	.83	.42	.21	.11	.05	.025	.013
Clotting time in sec (avg of 9 exp.)	13±2.9	16±2.8	30±5.2	58±6.5	160±35	‡	‡

B. *Action of enzymes at 38°C.*

Enzyme	Plasmin ("Fibrinolysin")		Trypsin		Chymotrypsin		$CHCl_3$-activated serum
No. of exp.	6	5	15	11	5	20	2
Conc. of enzyme per ml of final digestion mixture	670 γ = 0.2 U*	670 γ = 0.2 U*	1.7 to 3.3 γ	3.3 to 6.7 γ	12.5 to 16.7 γ	8.3 to 16.7 γ	0.17 ml
Minutes after start at 38°C	3	5 to 8	2 to 5	5 to 10	3	5 to 15	30, 45
% Destruction of clotting ability	96	>97	96	>97	94	>97	100†
% Hydrolysis to NP-compounds	1.4	3.0	1.8	3.5	9.0	14.8	5.0

* U = units according to Loomis(8).
† Lysis of fibrin clot.
‡ No clot.

centration of fibrinogen corresponded to a certain clotting time (Table IA). The percentage of proteolysis was estimated by converting optical density to mg of tyrosine-tryptophane, having the same ratio as in fibrinogen (5.8% tyrosine, 3.3% tryptophane). The results obtained by the above methods although approximations, furnish sufficient information about the comparative sensitivity of these test procedures. 15.0 ml of 0.4% fibrinogen† in veronal buffer of pH 7.35(7) was mixed with 3.0 ml of the enzyme solution in the same buffer and incubated at 37.5°C. At the start of the experiment, and after certain time intervals, the remaining fibrinogen was clotted by adding 1.0 ml of the digestion mixture to 0.5 ml of a 0.5% solution of thrombin (hemostatic globulin‡) in veronal buffer. In the experiments with trypsin, the thrombin solution employed contained 10 γ of crystalline trypsin inhibitor (soybean) per ml to stop the enzymatic action during the process of clotting. In separate experiments, it was found that the addition of this amount of inhibitor did not influence the clotting time. Simultaneously, the amount of non-protein tyrosine-tryptophane was estimated colorimetrically(5).

Table I demonstrates that using various proteolytic enzyme preparations,§ more than 97% of the clotting ability of fibrinogen is destroyed when only a very small percentage is hydrolyzed to non-protein compounds.

These results indicate: 1. The disintegration of fibrinogen to non-clottable products is by far a more sensitive indicator than the proteolytic disintegration to non-protein compounds. 2. The very first attack of these enzymes on fibrinogen is directed towards those groups which are responsible for the process of clot formation.

C. *Comparative proteolytic effects of trypsin and plasmin on casein and fibrinogen.* The possible identity of the serum proteolytic enzyme ("serum tryptase") with the fibrinolytic-fibrinogenolytic enzyme "plasmin" is indicated by the above results. This assumption is further strengthened by results of a preliminary comparative study of the proteolytic activity of trypsin and plasmin, under experimental conditions mentioned above, using casein and fibrinogen as a substrate. It was observed that the effect of 1 γ of crystalline trypsin (approx. 0.2 fibrinolytic units) in splitting casein to non-protein compounds equalled that of about 200 γ (0.06 fibrinolytic units)(8) of the "fibrinolysin" preparation employed. It should be emphasized that this result was obtained with a plasmin preparation, the absolute purity of which was not known. The same relation was found with fibrinogen as a substrate. The rate of degradation, produced by both enzymes, was about 4 to 5 times higher for casein than for fibrinogen as indicated by the amount of tyrosine-tryptophane liberated. About the same ratio was observed for chloroform-activated serum.

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† Preparation of bovine origin, containing 40-50% citrate, obtained from Armour Laboratories, Chicago, Ill.

7. Clark, D. G. C., Cliffton, E. E., and Newton, B. L., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 276.

‡ We wish to express our appreciation to Dr. H. D. Piersma of Lederle Laboratories, Pearl River, N. Y., for generously supplying us with this preparation.

§ We wish to express our appreciation to Dr. E. C. Loomis of Parke, Davis and Co., Detroit, Mich., for generously supplying us with various fibrinolysin preparations used in these studies.

D. *Fibrinolytic and antifibrinolytic activity of blood of rats in "tourniquet shock" and after epinephrine administration.* On the basis of the above results, an attempt was made to use the destruction of the clotting ability of fibrinogen as a test method for fibrinolytic activity of blood of rats, which had been subjected to "tourniquet shock." However, this was found unsatisfactory, as it is very difficult and often impossible to avoid the formation of thrombin, and consequently clot formation in the blood from rats in "tourniquet shock" while the blood is being drawn into the syringe. Therefore, the method of MacFarlane and Pilling(9), which measures the dissolution time of a fibrin clot, was employed for the determination of fibrinolytic activity. However, this method allows only an arbitrary expression of activity, which in this case was in terms of the greatest dilution of the plasma that will produce lysis (within 24 hours at 37°C) of the clot formed in a 0.018% solution of fibrinogen. Antifibrinolytic activity was determined according to Guest, Daly, Ware and Seegers(10,11). Armour's bovine fibrinogen was used as substrate. The required amount of thrombin (hemostatic globulin, Lederle) was added in 0.1 ml of saline, giving a final reaction volume of 0.5 ml. The inactivation of plasmin by plasma was carried out at room temperature for 60 minutes and the determination of the residual plasmin at 37.5°C. It was found that the small air bubbles, entrapped in the fibrin clot, serve well as an indicator for the dissolution of the fibrin clot. By observation through a magnifying glass and under proper illumination, the time at which the majority of the rapidly released air bubbles reaches the meniscus of the reaction mixture was taken as the end point of the dissolution of the fibrin clot. Calculations of antifibrinolytic activity were essentially identical with those of Guest, Daly, Ware and Seegers(10,11).

Table II shows that fibrinolytic activity was demonstrable in the plasma of rats in "tourniquet shock," from 15 to 90 minutes after release of the tourniquets. The percentage of positive cases was comparatively low. In separate experiments with the "fibrinolysin" preparation mentioned previously, it was found that the amount of plasmin in the blood of rats in "tourniquet shock" was of the order of 0.009 to 0.018 fibrinolytic units in 1 ml of the 1:64 diluted plasma. In the blood of normal control rats, no fibrinolytic activity could be detected. Table II shows that antifibrinolytic activity in rat plasma gradually decreases during "tourniquet shock."

Since it is known that epinephrine is involved in the condition of stress, the effect of epinephrine administration on the fibrinolytic and antifibrinolytic titer was studied. Rats were injected intraperitoneally with 0.50 mg of epinephrine and the fibrinolytic and antifibrinolytic level of plasma determined after certain time intervals.

Fibrinolytic activity could be demonstrated shortly after injection of epinephrine (Table III). A maximum was reached after about 20 minutes, while after 45 minutes no activity was detectable. The actual plasmin values have been found to be, on the average, about

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8. Loomis, E. C., and George, C., Jr., *Arch. Biochem.*, 1947, v12, 1.

9. MacFarlane, R. G., and Pilling, J., *Lancet*, 1946, v2, 562.

10. Guest, M. M., Ware, A. G., and Seegers, W. H., *Am. J. Physiol.*, 1947, v150, 661.

11. Guest, M. M., Daly, B. M., Ware, A. G., and Seegers, W. H., *J. Clin. Invest.*, 1948, v27, 785.

TABLE II. Fibrinolytic and Antifibrinolytic Activity in Plasma of Rats with Tourniquets on Both Hind Legs for 4½ Hours.

Time after release of tourniquet, min.	Fibrinolytic activity			Antifibrinolytic activity	
	No. of animals used	>.009 to .018 fibrinolytic units in 1 ml plasma (1:64) in No. of animals	% of animals	No. of animals used	Antifibrinolytic units per 1 ml of plasma (avg)
0	9	0	0	5	164
15	7	1	14	—	—
30	23	3	13	4	156
45	37	9	24	15	153
60	24	4	17	4	146
90	10	2	20	14	126
120	6	0	0	—	—
180	—	—	—	12	131
300	—	—	—	8	139
Control normal	42	0	0	25	174

TABLE III. Fibrinolytic and Antifibrinolytic Activity in Plasma of Rats After Epinephrine Injection (0.50 mg).

Time after epinephrine injection, min.	Fibrinolytic activity			Antifibrinolytic activity	
	No. of animals used	>.018 to .036 fibrinolytic units in 1 ml plasma (1:64) in No. of animals	% of animals	No. of animals used	Antifibrinolytic units per 1 ml of plasma (avg)
5	2	0	0	—	—
10	7	1	14	—	—
15	9	4	44	3	149
20	12	8	66	2	131
30	9	4	44	5	128
45	5	0	0	6	115
60	4	0	0	2	120
90	5	0	0	13	128
120	5	0	0	3	133
180	—	—	—	11	134
240	2	0	0	2	126
300	—	—	—	14	151
360	2	0	0	—	—
480	—	—	—	10	172
Control normal	42	0	0	25	174

twice as high as in the positively reacting blood of rats in "tourniquet shock," based on the dilution up to which fibrinolysis could be observed. The antifibrinolytic titer in plasma was decreased, reaching the lowest values after the disappearance of the fibrinolytic activity. The sharp initial decline, found 45 minutes after epinephrine administration, is followed by a slow rise of antifibrinolytic activity, reaching normal levels after about 8 hours. The similarity in the counterplay between plasmin and plasmin inhibitor in "tourniquet shock" and after epinephrine administration is apparent. The appearance and disappearance of fibrinolytic activity within a few minutes after epinephrine injection may suggest that plasmin is rapidly inactivated or eliminated or taken up by various tissues leading to an increased protein breakdown in these tissues.

Apparently, fibrinolytic activity could not be produced in rats as consistently as in humans(12). The dependability of demonstrating fibrinolytic activity may be limited due to a possible loss of plasmin from the plasma since it is strongly adsorbed by fibrin.

Summary and conclusions. No change in proteolytic activity could be demonstrated in the serum of rats in "tourniquet shock," using

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12. Biggs, R., MacFarlane, R. G., and Pilling, J., *Lancet*, 1947, v1, 402.

the degree of liberation of tyrosine-tryptophane from a casein substrate as an index of proteolytic activity. The antitryptic titer in the same animals, measured on crystalline trypsin with casein as a substrate, was slightly lowered, compared to normal. Comparison of the fibrinogenolytic and proteolytic activities of trypsin, plasmin and the chloroform activated serum enzyme indicated parallelism between these enzymes. This similarity further substantiates the claim that plasmin can act as a proteolytic enzyme and may well play a significant role in the process of protein breakdown. It was found not to be feasible to use the destruction of the clotting ability of fibrinogen as a test method for fibrinolytic activity in blood of rats, subjected to "tourniquet shock." Fibrinolytic activity and reduced antifibrinolytic activity could be demonstrated in the plasma of rats in "tourniquet shock" and after epinephrine administration to normal rats. The fibrinolytic activity observed may be due either to an actual decrease in the concentration of the plasmin inhibitor or to an augmentation of the plasmin content. The recognition of plasmin and plasmin inhibitor as balancing components of a proteolytic system existing in normal blood greatly facilitates an approach to the problem of the mechanism of protein breakdown.

Received October 30, 1950. P.S.E.B.M., 1950, v75.

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